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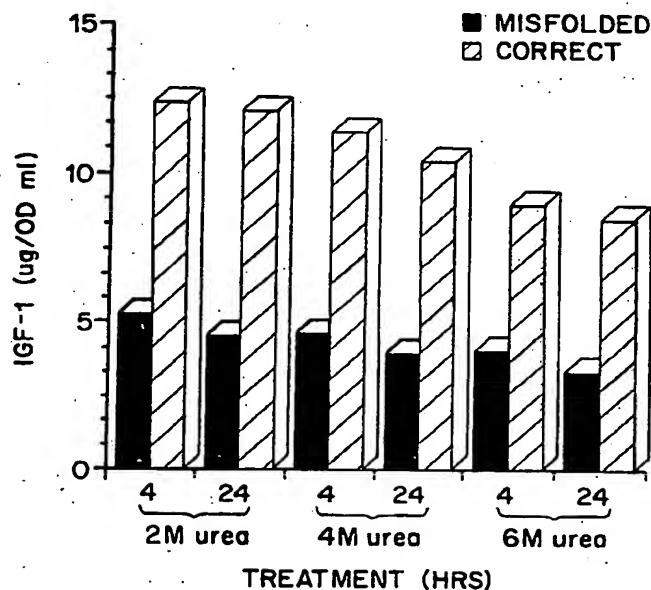
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**(54) Title:** METHOD FOR REFOLDING IGF-I TO ACTIVE CONFORMATION**(57) Abstract**

A method for refolding insoluble, improperly folded IGF-I is provided, wherein the IGF-I, precipitated from prokaryotic host cells, is concurrently solubilized, unfolded, and refolded into a biologically active conformation in a single buffer. The buffer contains reducing agent and chaotropic agent to solubilize the IGF-I at concentrations sufficiently low to allow solubilization and refolding to occur. Also provided is a triple-protease deficient *E. coli* host suitable for use in the process.

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## METHOD FOR REFOLDING IGF-I TO ACTIVE CONFORMATION

5

Background of the InventionField of the Invention

10 This invention relates to an efficient method for refolding insulin-like growth factor-I (IGF-I) that has been produced in heterologous host cells and is present in these cells as clumps of insoluble protein.

Description of Related Art

15 The production of large quantities of relatively pure, biologically active polypeptides and proteins is important economically for the manufacture of human and animal pharmaceutical formulations, enzymes, and other specialty chemicals. For production of many proteins, recombinant DNA techniques have become the method of choice because large quantities of exogenous proteins can be expressed in bacteria and other host cells free of other contaminating proteins.

20 Producing recombinant protein involves transfecting host cells with DNA encoding the protein and growing the cells under conditions favoring expression of the recombinant protein. The prokaryote *E. coli* is favored as host because it can be made to produce recombinant proteins in high yields. Numerous U.S. patents on general bacterial expression of recombinant-DNA-encoded proteins exist, including U.S. Pat. No. 4,565,785 on a recombinant DNA molecule comprising a bacterial gene for an extracellular or periplasmic carrier protein and non-bacterial gene; 4,673,641 on coproduction of a foreign polypeptide with an aggregate-forming polypeptide; 4,738,921 on an expression vector with a trp promoter/operator and trp LE fusion with a polypeptide such as IGF-I; 4,795,706 on expression control sequences to include with a foreign protein; and 4,710,473 on specific circular DNA plasmids such as those encoding IGF-I.

30 Under some conditions, certain heterologous proteins expressed in large quantities from bacterial hosts are precipitated within the cells in dense masses, recognized as bright spots visible within the enclosure of the cells under a phase contrast microscope at magnifications down to 1000 fold. These clumps of precipitated proteins are referred to as "refractile bodies," and constitute a significant portion of the total cell protein. Brems *et al.*, Biochemistry, 24: 7662 (1985). On the other hand, the clumps of protein may not be visible under the phase contrast microscope, and the expression "inclusion body" is often used to refer to the aggregates of protein whether visible or not under the phase contrast microscope.

40 Recovery of the protein from these bodies has presented numerous problems, such as how to separate the protein encased within the cell from the cellular material and proteins harboring it, and how to recover the inclusion body protein in biologically active form. The

recovered proteins are often biologically inactive because they are folded into a three-dimensional conformation different from that of active protein. For example, recombinant IGF-I that has disulfide bonds formed between cysteine pairs different from the pairs found in the disulfide bonds of native IGF-I has significantly reduced biological activity. Raschdorf  
5 *et al.*, Biomedical and Environmental Mass Spectroscopy, 16: 3-8 (1988). Misfolding occurs either in the cell or during the isolation procedure. Methods for refolding the proteins into the correct, biologically active conformation are essential for processing functional proteins.

Protein folding is influenced by the nature of the medium containing the protein and by a combination of weak attractive or repellent intramolecular forces involved in hydrogen  
10 bonding, ionic bonding, and hydrophobic interactions. When pairs of cysteine residues are brought into close proximity as the peptide backbone folds, strong covalent disulfide bonds form between cysteine residues, serving to lock the tertiary conformation in place. Refolding protocols have been designed to break incorrect disulfide bonds, block random disulfide bonding, and allow refolding and correct disulfide bonding under conditions favorable to the  
15 formation of active conformer.

It has been found that the soluble proportion of high-level expressed protein in *E. coli* has been dramatically increased by lowering the temperature of fermentation to below 30°C. A considerable fraction of various foreign proteins, i.e., human IFN- $\alpha$ 2, IFN- $\gamma$ , and murine MX protein [Schein and Noteborn, Bio/Technology, 6: 291-294 (1988)] and human IFN- $\beta$   
20 [Mizukami *et al.*, Biotechnol. Lett., 8: 605-610 (1986)], stayed in solution. This procedure represents an alternative to renaturation of proteins recovered from refractile bodies, but requires an expression system that is efficiently induced at temperatures below 30°C. The procedure is therefore not effective for all proteins.

One series of techniques for recovering active protein from inclusion bodies involves  
25 solubilizing the inclusion bodies in strongly denaturing solutions and then optionally exchanging weakly denaturing solutions for the strongly denaturing solutions (or diluting the strong denaturant), or using molecular sieve or high-speed centrifugation techniques. Such recovery methods, described, e.g., in U.S. Pat. Nos. 4,512,922; 4,518,256; 4,511,502; and 4,511,503, are regarded as being universally applicable, with only minor modifications, to the  
30 recovery of biologically active recombinant proteins from inclusion bodies. These methods seek to eliminate random disulfide bonding prior to coaxing the recombinant protein into its biologically active conformation through its other stabilizing forces.

In one such method, the denatured protein desired to be refolded is further purified under reducing conditions that maintain the cysteine moieties of the protein as free sulfhydryl  
35 groups by supplying a reducing agent throughout all the purification steps. This allows the protein to refold itself under the conditions of purification in the absence of incorrect disulfide bond formation. The reducing agent is then diluted into an aqueous solution to enable the refolded protein to form the appropriate disulfide bonds in the presence of air or some other

oxidizing agent. This enables refolding to be easily incorporated into the overall purification process and works optimally for recombinant proteins having relatively uncomplicated tertiary structures in their biologically active forms.

In a second approach in this series, refolding of the recombinant protein takes place in the presence of both the reduced (R-SH) and oxidized (R-S-S-R) forms of a sulfhydryl compound. This allows free sulfhydryl groups and disulfides to be formed and reformed constantly throughout the purification process. The reduced and oxidized forms of the sulfhydryl compound are provided in a buffer having sufficient denaturing power that all of the intermediate conformations of the protein remain soluble in the course of the unfolding and refolding. Urea is suggested as a suitable buffer medium because of its apparent ability to act both as a sufficiently weak denaturing agent to allow the protein to approximate its correct conformation and as a sufficiently strong denaturant that the refolding intermediates maintain their solubility. This approach works best where the recombinant inclusion body proteins of interest have relatively uncomplicated folding patterns.

The third alternative in this series, used in more difficult refolding situations, is designed to break any disulfide bonds that may have formed incorrectly during isolation of the inclusion bodies and then to derivatize the available free sulfhydryl groups of the recombinant protein. This objective is achieved by sulfonating the protein to block random disulfide pairings, allowing the protein to refold correctly in weak denaturant, and then desulfonating the protein, which protocol favors correct disulfide bonding. The desulfonation takes place in the presence of a sulfhydryl compound and a small amount of its corresponding oxidized form to ensure that suitable disulfide bonds will remain intact. The pH is raised to a value such that the sulfhydryl compound is at least partially in ionized form to enhance nucleophilic displacement of the sulfonate.

These refolding protocols, while practical for their universal utility, have not been shown necessarily to be maximally efficient with, for example, recombinant IGF-I.

Enhancement of selected disulfide pairings by adding 50% methanol to buffer at low ionic strength has been reported by G.H. Snyder, J. Biol. Chem., **259**: 7468-7472 (1984). The strategy involves enhancing formation of specific disulfide bonds by adjusting electrostatic factors in the medium to favor the juxtaposition of oppositely charged amino acids that border the selected cysteine residues. See also Tamura *et al.*, abstract and poster presented at the Eleventh American Peptide Symposium on July 11, 1989 advocating addition of acetonitrile, DMSO, methanol, or ethanol to improve the production of the correctly folded IGF-I. U.S. Pat. No. 4,923,967 and EP 361,830 describe a protocol for solubilizing and sulphytolysing refractile protein in denaturant, then exchanging solvent to precipitate the protein. The protein is resolubilized in denaturant and allowed to refold in the presence of reducing agent. The multiple steps required to achieve correct folding are time-consuming.

The recovery of the biological activity requires a carefully monitored renaturation procedure and may be very difficult depending on the protein in question. A number of publications have appeared that report refolding attempts for individual proteins that are produced in bacterial hosts or are otherwise in a denatured or non-native form. For example, formation of a dimeric, biologically active M-CSF after expression in *E. coli* is described in WO 88/8003 and by Halenbeck *et al.*, Biotechnology, 7: 710-715 (1989). The procedures described involve the steps of initial solubilization of M-CSF monomers isolated from inclusion bodies under reducing conditions in a chaotropic environment comprising urea or guanidine hydrochloride, refolding achieved by stepwise dilution of the chaotropic agents, and final oxidation of the refolded molecules in the presence of air or a redox-system.

Reasonable recovery after renaturation has been reported for several proteins such as interleukin-2 (IL-2) [Tsuji *et al.*, Biochemistry, 26: 3129-3134 (1987); WO 88/8849], growth hormone from various sources [George *et al.*, DNA, 4: 273-281 (1984); Gill *et al.*, Bio/Technology, 3: 643-646 (1985); Sekine *et al.*, Proc. Natl. Acad. Sci. USA, 82: 4306-4310 (1985); U.S. Pat. No. 4,985,544, the lattermost reference involving adding a denaturing agent and reducing agent to solubilize the protein, removing the reducing agent, oxidizing the protein, and removing the denaturing agent], prochymosin [Green *et al.*, J. Dairy Res., 52: 281-286 (1985)], urokinase [Winkler *et al.*, Bio/Technology, 3: 990-1000 (1985)], somatotropin [U.S. Pat. No. 4,652,630, whereby urea is used for solubilization, and a mild oxidizing agent is then used for refolding], and tissue-plasminogen activator [Rudolph *et al.*, in "623rd Biochem. Soc. Meeting," Canterbury (1987)]. See also Marston, Biochemical J., 240: 1 (1986).

Where the efficiency of recovery has been reported, up to 40% active foreign protein has been obtained. See, e.g., Boss *et al.*, Nucl. Acids Res., 12: 3791-3806 (1984); Cabilly *et al.*, Proc. Natl. Acad. Sci. USA, 81: 3273-3277 (1984); Marston *et al.*, Bio/Technology, 2: 800-804 (1984); and Rudolph *et al.*, *supra*. However, such yields may not be acceptable if the protein is costly to produce and must be made in commercial quantities. Additional representative literature on refolding of non-native proteins derived from different sources include a report that IL-2 and interferon- $\beta$  (IFN- $\beta$ ) have been refolded using SDS for solubilization and Cu<sup>+2</sup> ions as oxidation promoters of the fully reduced proteins [U.S. Pat. No. 4,572,798]. A process for isolating recombinant refractile proteins as described in U.S. Pat. No. 4,620,948 involves using strong denaturing agents to solubilize the proteins, reducing conditions to facilitate correct folding, and denaturant replacement in the presence of air or other oxidizing agents to reform the disulfide bonds. The proteins to which the process can be applied include urokinase, human, bovine, and porcine growth hormone, interferon, tissue-type plasminogen activator, FMD coat protein, prorennin, and the src protein.

A method for renaturing unfolded proteins including cytochrome c, ovalbumin, and trypsin inhibitor by reversibly binding the denatured protein to a solid matrix and stepwise renaturing it by diluting the denaturant is disclosed in WO

86/5809. A modified monomeric form of human platelet-derived growth factor (PDGF) expressed in *E. coli* has been S-sulfonated during purification to protect thiol moieties and then dimerized in the presence of oxidizing agents to yield the active protein. Hoppe *et al.*, Biochemistry, 28: 2956 (1989).

Additionally, EP 433,225 published 19 June 1991 discloses a process for producing dimeric biologically active transforming growth factor- $\beta$  protein or a salt thereof wherein the denatured monomeric form of the protein is subjected to refolding conditions that include a solubilizing agent such as mild detergent, an organic, water-miscible solvent, and/or a phospholipid. See also Bowden *et al.*, Bio/Technology, 9: 725 (1991) on  $\beta$ -lactamase cytoplasmic and periplasmic inclusion bodies, and Samuelsson *et al.*, Bio/Technology, 9: 731 (1991) on refolding of human interferon-gamma mutants. For general review articles, see Marston, Biochem. J., 240: 1-12 (1986); Mitraki and King, Bio/Technology, 7: 690 (1989); Marston and Hartley, Methods in Enzymol., 182: 264-276 (1990); Wetzel, "Protein Aggregation *In Vivo*: Bacterial Inclusion Bodies and Mammalian Amyloid," in Stability of Protein Pharmaceuticals: In Vivo Pathways of Degradation and Strategies for Protein Stabilization, Ahern and Manning (eds.), Plenum Press, 1991; and Wetzel, "Enhanced Folding and Stabilization of Proteins by Suppression of Aggregation *In Vitro* and *In Vivo*," in Protein Engineering—A Practical Approach, Rees, A.R. *et al.* (eds.), IRL Press at Oxford University Press, Oxford, 1991.

Several literature references exist on the production of IGF-I in bacteria. These include EP 128,733 published 19 December 1984 and EP 135,094 published 27 March 1985, which address expression of IGF-I in bacteria. EP 288,451 addresses use of lamB or ompF signal to secrete IGF-I in bacteria; Obukowicz *et al.*, Mol. Gen. Genet., 215: 19-25 (1988) and Wong *et al.*, Gene, 68: 193-203 (1988) teach similarly. EP 286,345 discloses fermentation of IGF-I using a lambda promoter.

In addition, methods have been suggested for preparing IGF-I as a fusion protein. For example, EP 130,166 discloses expression of fusion peptides in bacteria, and EP 155,655 (U.S. Pat. No. 5,019,500) and EP 219,814 disclose a fusion of IGF-I with a protective polypeptide for expression in bacteria. EP 264,074 discloses a two-cistronic met-IGF-I expression vector with a protective peptide of 500-50,000 molecular weight (see also U.S. Pat. No. 5,028,531 and Saito *et al.*, J. Biochem., 101: 1281-1288 (1987)). Other IGF-I fusion techniques that have been reported include fusion with protective peptide from which a rop gene is cut off [EP 219,814], IGF-I multimer expression [Schulz *et al.*, J. Bacteriol., 169: 5385-5392 (1987)], fusion of IGF-I with LH protein through a chemically cleavable methionyl or tryptophan residue at the linking site [Saito *et al.*, J. Biochem., 101: 123-134



(1987)], and fusion with superoxide dismutase [EP 196,056]. Niwa *et al.*, Ann. NY Acad. Sci., **469**: 31-52 (1986) discusses the chemical synthesis, cloning, and successful expression of genes for IGF-I fused to another polypeptide.

5 These methods utilizing fusion proteins, however, generally require a relatively long leader sequence and are directed to improving expression of the inclusion body protein, not to improving refolding of the denatured recombinant protein. Int. Pub. No. WO 91/02807 published 7 March 1991 describes a method for refolding recombinant IGF-I that involves cloning the IGF-I gene with a positively charged leader sequence prior to transfecting the DNA into the host cell. The additional positive charge on the amino terminus of the recombinant  
10 IGF-I allows correct refolding when the solubilized protein is stirred for 2-16 hours in denaturant solution. Following refolding, the leader sequence is cleaved and the active recombinant protein is purified. This multistep process is burdensome, requiring additional materials and effort to clone a heterologous leader sequence in front of the IGF-I gene and then to remove the leader sequence from the purified protein. Additionally, the 30-50% yield  
15 of active conformer, using this method, is unremarkable.

Another method for facilitating *in vitro* refolding of recombinant IGF-I involves using a solubilized affinity fusion partner consisting of two IgG-binding domains (ZZ) derived from staphylococcal protein A. Samuelsson *et al.*, Bio/Technology, **9**: 363 (1991). While this method, which uses the protein A domain as a solubilizer of misfolded and multimeric IGF-I,  
20 results in higher yields of IGF-I without the use of denaturing agents or redox chemicals, it involves the extra steps of fusing onto the IGF-I gene a separate gene and removing the polypeptide encoded by that gene after expression of the fusion gene.

As regards the bacterial hosts that may be utilized for fermentation processes, WO 88/05821 published 11 August 1988 discloses a method of isolating a mutant strain of *E. coli* having a defective periplasmic protease. WO 89/02465 published 23 March 1989 discloses a process for production of a polypeptide comprising direct expression of the polypeptide in bacterial host cells using an inducible expression system in combination with a protease-deficient bacterial host system, including a host deficient in two proteases. WO  
25 85/03949 published 12 September 1985 discloses bacterial cell strains carrying specific mutations within their DNA sequences that cause the cells to exhibit a reduced capacity for degrading foreign products due to reduced expression of cellular proteases, with a htpR lon *E. coli* host exemplified. WO 89/10976 published 16 November 1989 discloses protease-deficient gram-positive bacteria and their use as host organisms for producing recombinant proteins. In addition, Buell *et al.*, Nucl. Acids Res., **13**: 1923-1938 (1985) discloses use of  
30 an *E. coli* host mutated at lon and htpR to produce IGF-I.

35 There is a need in the art for a simple, one-step, efficient protocol for refolding insoluble, misfolded IGF-I into the correct conformation so that the biological activity of the IGF-I can be restored.

Accordingly, it is an object of the present invention to provide such procedure for reactivating, in one step, misfolded IGF-I recovered from inclusion bodies formed in prokaryotic cells, allowing for recovery of biologically active IGF-I at low cost and high yield.

It is a particularly preferred object to provide a one-step solubilization and refolding procedure to reactivate misfolded recombinant IGF-I precipitated in the periplasmic space of bacterial host cells.

It is another object to provide protease-deficient *E. coli* hosts that are particularly suited for the solubilization and refolding process herein.

These and other objects will be apparent to those of ordinary skill in the art.

#### 10 Summary of the Invention

Accordingly, the present invention provides a method for reactivating insoluble, misfolded IGF-I present in prokaryotic host cells by isolating the insoluble IGF-I and incubating it in an alkaline buffered solution comprising no more than 3 M chaotropic agent and the minimum amount of reducing agent necessary for solubilization, under conditions of IGF-I concentration and incubation temperature and time so that unfolding and refolding to the active conformation take place concurrently with solubilization in the same buffer.

Preferably, the IGF-I is isolated by exposing the cells to a buffer of suitable ionic strength to solubilize most host polypeptides, but not the insoluble IGF-I, disrupting the cells to form a soluble and an insoluble fraction, and isolating the insoluble IGF-I fraction as by centrifugation.

The use of minimal amounts of chaotropic agent and reducing agent allows breaking of disulfide bonds so that misfolded conformers can correctly refold under mild conditions. In addition, the entire solubilization and refolding process takes place in one simple incubation step without the need to remove any chaotropic or reducing agent or to add redox buffer or other agent.

In another aspect, the invention provides an *E. coli* strain having the complete genotype *tonAΔ ptr3 phoAΔE15 Δ(largF-lac)169* in a W3110 background, and the strains additionally having the genotype *ompTΔ* or *ompTΔ degP41*. The invention further provides for such strains that are transformed with a vector encoding IGF-I. These strains can be utilized in the process of this invention.

#### 30 Brief Description of the Drawings

Figure 1 illustrates the lineage of *E. coli* W3110 host strain 27C7.

Figures 2A and 2B depict the schemes for the mutation of the *tonA* and *phoA* genes, respectively, in *E. coli* strain W3110.

Figure 3 depicts in detail the construction for the elimination of the *tonA* gene from W3110.

Figure 4 depicts the construction of plasmid pLS32, an intermediate plasmid in preparing pLS32Tsc, which contains a gene encoding IGF-I.

Figure 5 depicts the construction of pAPlamB, another intermediate in preparing pLS32Tsc.

Figure 6 depicts the construction of pLS32lamB, still another intermediate in the construction of pLS32Tsc.

5 Figure 7 depicts the construction of pLS33lamB, yet another intermediate in the preparation of pLS32Tsc.

Figure 8 depicts the construction of pLS33Tsc, yet another intermediate in the preparation of pLS32Tsc.

Figure 9 depicts the construction of pLS32Tsc from pLS33Tsc and pLS32lamB.

10 Figure 10 depicts the nucleotide sequence of the expression cassette and amino acid sequence encoding the lamB signal sequence and the IGF-I in plasmid pLS32Tsc.

Figures 11A and 11B show the effect on IGF-I RIA titers of various pH values and buffers used in solubilizing IGF-I.

15 Figure 12 depicts a graph of resultant IGF-I titer as a function of the concentration of IGF-I present, with a constant IGF-I/DTT ratio.

Figures 13A, 13B, and 13C show the effect on IGF-I RIA titers of duration time in the solubilizing buffer for refolding, with Fig. 13A using a pH of 8.2 with no methanol, Fig. 13B using a pH of 10.5 with no methanol, and Fig. 13C using a pH of 10.5 with methanol.

20 Figures 14A and 14B show IGF-I titer and correct:misfolded IGF-I as a function of the concentration of dithiothreitol (DTT) and  $\beta$ -mercaptoethanol (BME), respectively, in the solubilizing buffer.

Figure 15A shows correct IGF-I formation as a function of IGF-I added for IGF-I solubilized using IGF-I/DTT = 0.11, 1 mM DTT, and 2 mM DTT. Figures 15B, 15C, and 15D show IGF-I titer and correct:misfolded IGF-I as a function of IGF-I added for IGF-I solubilized  
25 using a constant IGF-I/DTT = 0.11, 2 mM DTT, and 1 mM DTT, respectively.

Figure 16 shows the effect of dilution to 2 M urea of more concentrated urea solutions on folding, where the slashed bars are correctly folded IGF-I and the solid black bars are misfolded IGF-I, examining original urea concentrations of 2 M, 4 M, and 6 M at 4 hours (diluted and incubated for another 5 hours) and 24 hours (undiluted).

### 30 Description of the Preferred Embodiments

#### A. Definitions.

As used herein, "insoluble, misfolded IGF-I" refers to precipitated or aggregated IGF-I that is contained within the periplasm of prokaryotic host cells, or is otherwise prokaryotic host cell associated, and assumes a biologically inactive conformation with mismatched or  
35 unformed disulfide bonds. The insoluble IGF-I is preferably, but need not be, contained in refractile bodies, i.e., it may or may not be visible under a phase contrast microscope.

The term "conformers" refers to polypeptides recovered from inclusion bodies containing recombinant IGF-I that differ only in intramolecular disulfide bonding. IGF-I is 70

amino acids long and has six cysteine residues that form intramolecular disulfide bonds. The correct, active conformer has disulfide bonds between amino acid residues C6-C48, C47-C52, and C18-C61. The other main polypeptide recovered from such inclusion bodies is a misfolded, biologically inactive conformer having disulfide bonds between amino acid residues C6-C47, C48-C52, and C18-C61.

As used herein, "chaotropic agent" refers to a compound that, in a suitable concentration in aqueous solution, is capable of changing the spatial configuration or conformation of proteins through alterations at the surface thereof so as to render the IGF-I up to about 90% soluble in the aqueous medium. The alterations may occur by changing, e.g., the state of hydration, the solvent environment, or the solvent-surface interaction. The concentration of chaotropic agent will directly affect its strength and effectiveness. A strong chaotropic agent is a compound which, in solution, will effectively unfold a protein present in the solution. The unfolding will be relatively extensive, but reversible. A moderate chaotropic agent is a compound which, in solution, permits partial folding of a protein from whatever conformed conformation the protein has assumed through intermediates soluble in the solution, into the spatial conformation in which it finds itself when operating in its active form under endogenous or homologous physiological conditions.

As used herein, "IGF-I" refers to insulin-like growth factor from any species, including bovine, ovine, porcine, equine, and preferably human, in native sequence or in variant form and recombinantly produced. Preferred herein, IGF-I is cloned, and expressed, e.g., by the process described in EP 128,733 published December 19, 1984.

As used herein, "reducing agent" refers to a compound or material which, in a suitable concentration in aqueous solution, maintains sulfhydryl groups in the reduced state and reduces disulfide intra- or intermolecular bonds.

As used herein, "buffered solution" refers to a solution which resists changes in pH by the action of its acid-base conjugate components.

As used herein, "hydrophobic agent" refers to a nonpolar solute that, when added to aqueous solution, increases the hydrophobicity of the solution.

#### B. Modes for Carrying Out the Invention

Insoluble, misfolded IGF-I is isolated from host cells in a suitable isolation buffer by any appropriate technique, e.g. one involving exposing the cells to a buffer of suitable ionic strength to solubilize most host proteins, but in which aggregated IGF-I is substantially insoluble, disrupting the cells so as to release the inclusion bodies and make them available for recovery by, for example, centrifugation. This technique is well known, and is described, for example, in U.S. Pat. No. 4,511,503. Briefly, the cells are suspended in the buffer (typically at pH 5 to 9, preferably about 6 to 8, using an ionic strength of about 0.01 to 2 M, preferably 0.1 to 0.2 M). Any suitable salt, including NaCl, is useful to maintain a sufficient ionic strength value. The cells, while suspended in this buffer, are then disrupted by lysis

using techniques commonly employed such as, for example, mechanical methods, e.g., a Manton-Gaulin press, a French press, or a sonic oscillator, or by chemical or enzymatic methods.

5 Examples of chemical or enzymatic methods of cell disruption include spheroplasting, which entails the use of lysozyme to lyse the bacterial wall [H. Neu et al., Biochem. Biophys. Res. Comm., 17: 215 (1964)], and osmotic shock, which involves treatment of viable cells with a solution of high tonicity and with a cold-water wash of low tonicity to release the polypeptides [H. Neu et al., J. Biol. Chem., 240(9): 3685-3692 (1965)]. A third method, described in U.S. Pat. No. 4,680,262 issued July 14, 1987, involves contacting the  
10 transformed bacterial cells with an effective amount of a lower alkanol having 2 to 4 carbon atoms for a time and at a temperature sufficient to kill and lyse the cells.

After the cells are disrupted, the suspension is typically centrifuged at low speed, generally around 500 to 15,000 x g, preferably about 12,000 x g, in a standard centrifuge for a sufficient time that depends on volume and centrifuge design, usually about 10 minutes  
15 to 0.5 hours, preferably 12,000 x g for 10 minutes. The time may be substantially reduced in a disc stack type centrifuge. The resulting pellet contains substantially all of the insoluble IGF-I fraction, but if the cell disruption process is not complete, it may also contain intact cells or broken cell fragments. Completeness of cell disruption can be assayed by resuspending the pellet in a small amount of the same buffer solution and examining the  
20 suspension with a phase contrast microscope if the IGF-I is contained in refractile bodies. The presence of broken cell fragments or whole cells indicates that further sonication or other means of disruption is necessary to remove the fragments or cells and the associated non-refractile polypeptides. After such further disruption, if required, the suspension is again centrifuged and the pellet recovered, resuspended, and reexamined. The process is repeated  
25 until visual examination reveals the absence of broken cell fragments in the pelleted material or until further treatment fails to reduce the size of the resulting pellet.

The above process can be employed whether the insoluble protein is intracellular or in the periplasmic space. The preferred conditions given herein for isolating IGF-I are directed particularly to inclusion bodies precipitated in the periplasmic space.

30 The isolated insoluble, misfolded IGF-I is then incubated in alkaline buffer containing the minimum amount of chaotropic agent and reducing agent necessary substantially to solubilize the IGF-I and allow refolding. This incubation takes place under conditions of IGF-I concentration, incubation time, and incubation temperature that will allow solubilization of IGF-I, and unfolding and refolding of IGF-I to occur concurrently in the alkaline buffer.

35 Measurement of the degree of solubilization of the IGF-I in the buffer is suitably carried out by turbidity determination, by analyzing IGF-I fractionation between the supernatant and pellet after centrifugation on reduced SDS gels, by protein assay (e.g., the Bio-Rad protein assay kit), or by HPLC. The degree of refolding is suitably determined by the RIA titer of IGF-I

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or by HPLC analysis using *e.g.*, a Vydac C18 column, with increasing RIA titer or correctly folded IGF-I peak size directly correlating with increasing amounts of correct, biologically active IGF-I conformer present in the buffer. The incubation is carried out to maximize the ratio of correctly folded IGF-I conformer to misfolded IGF-I conformer recovered, as determined by RIA or HPLC.

The pH range of the buffer typically is at least about 7.5, with the preferred range being 7.5-10.5. Examples of suitable buffers that will provide a pH within this latter range include CAPSO (3-[Cyclohexylamino]-2-hydroxy-1-propanesulfonic acid), AMP (2-Amino-2-methyl-1-propanol), CAPS (3-[Cyclohexylamino]-1-propanesulfonic acid), CHES (2-[N-Cyclohexylamino]ethanesulfonic acid), TRIS (Tris(hydroxymethyl)aminomethane), and sodium acetate. The preferred buffer herein is CAPSO at about pH 8.5-10.5.

Examples of suitable reducing agents include DTT, BME, and cysteine. The minimal amount of reducing agent to be present in the buffer will depend mainly on the type of reducing agent and chaotropic agent, the type and pH of the buffer employed, the amount of oxygen entrained in or introduced to the solution, and the concentration of the IGF-I in the buffer. For example, with 0.5-1.5 mg/ml IGF-I in a buffered solution at pH 7.5-10.5 containing 1-4 M urea, the DTT concentration is at about 1-8 mM and the BME concentration is at about 0.2-2 mM. The preferred reducing agent is DTT at about 2-4 mM, BME at about 1-2 mM, or cysteine at about 2-4 mM.

Chaotropic agents suitable for practicing this invention include, *e.g.*, urea and salts of guanidine or thiocyanate, more preferably urea, guanidine hydrochloride, or sodium thiocyanate. The amount of chaotropic agent minimally necessary to be present in the buffer is no more than about 3 molar. The preferred chaotropic agent herein is urea at about 1.5-2.5 M, more preferably at about 2 M, or guanidine hydrochloride at about 1-3 M.

The conditions of incubation of the insoluble, misfolded IGF-I will generally be such that substantial or complete solubilization of the IGF-I will take place, as well as maximal refolding. The exact conditions will depend on, *e.g.*, the pH of the buffer and the types and concentrations of chaotropic and reducing agents. The reaction temperature must not be so high as to denature the IGF-I, and is therefore generally about 10-40°C. The incubation will generally be carried out for at least about 1 hour to effect concurrent solubilization and refolding. The reaction is preferably carried out at about 15-37°C, more preferably 20-30°C, for at least about 1 hour, more preferably 2-12 hours.

The concentration of the IGF-I in the buffered solution must be such that the IGF-I will be substantially solubilized and the ratio of correctly folded to misfolded conformer recovered will be maximized, as determined by HPLC. The exact amount to employ will depend, *e.g.*, on the concentrations and types of other ingredients in the buffered solution, particularly the IGF-I concentration, reducing agent, and the pH of the buffer. For example, the concentration of IGF-I may be increased at least three-fold without decreasing the ratio of correct to

misfolded conformer if the concentration of reducing agent, e.g. DTT, is concurrently increased, to maintain a ratio of IGF-I:DTT of from about 0.11 to 0.2. The preferred concentration of IGF-I (resulting in the maximum yield of correctly folded conformer) is in the range of 0.5-5.5 mg per ml, more preferably 1.5-5.0 mg/ml.

5 Addition of a hydrophobic agent to the buffer is generally suitable for increasing the yield of correctly folded conformer. Examples of suitable hydrophobic agents include organic solvents such as methanol, ethanol, DMSO (dimethylsulfoxide), and acetonitrile. Methanol and ethanol are effective as about 5-20% solutions, more preferably about 20%, and DMSO is effective as about a 40-50% solution.

10 Host cells that express the recombinant IGF-I abundantly in the form of inclusion bodies are prokaryotic cells. Suitable prokaryotes include bacteria, preferably eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, *Bacilli* such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescens*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Strain W3110 is a preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins, with examples of such hosts including *E. coli* W3110 strains 1A2, 27A7, 27B4, and 27C7, described further below.

20 The invention will be more fully understood by reference to the following examples which are intended to illustrate the invention but not to limit its scope. All literature and patent citations are expressly incorporated by reference.

#### 25 EXAMPLE I

##### Production and Isolation of rhIGF-I And Test for Its Solubility in Tris Buffer

###### i. Construction of host cell strain 27C7

30 The host used to produce recombinant human IGF-I in the fermentation described herein was a derivative of *E. coli* W3110, designated 27C7. The complete genotype of 27C7 is *tonAΔ ptr3 phoAΔE15 Δ(largF-lac)169 ompTΔ degP41kan<sup>r</sup>*. The derivation of strain 27C7 is diagrammed in Figure 1 and is described below. Strain 27C7 was deposited on October 30, 1991 in the American Type Culture Collection as ATCC No. 55,244.

35 Strain 27C7 was constructed in several steps using techniques involving transductions with phage P1kc, derived from P1 (J. Miller, Experiments in Molecular Genetics (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory, 1972)), and transposon genetics [Kleckner et al., J. Mol. Biol., **116**: 125-159 (1977)]. The starting host used was *E. coli* K12 W3110, which is a K12 strain that is F-, A- [Bachmann, Bact. Rev., **36**: 525-557 (1972); Bachman,

"Derivatives and Genotypes of Some Mutant Derivatives of *Escherichia coli* K-12," p. 1190-1219, In F.C. Niedhardt *et al.*, ed., Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology, vol. 2, American Society for Microbiology, Washington, D.C. (1987)).

First, the *tonA* gene (*fhuA*) [Kadner *et al.*, J. Bact., **143**: 256-264 (1980), Bachmann, Microbiol. Rev., **47**: 180-230 (1983), Bachman, "Linkage Map of *Escherichia coli* K-12," edition 7, p. 807-876, in F.C. Niedhardt *et al.*, ed., Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology, Vol. 2, American Society for Microbiology, Washington, D.C., 1987] was deleted from W3110 by the insertion and subsequent imprecise excision of a *Tn10* transposon into the *tonA* gene. This construction is diagrammed in Figures 2B and 3.

In the first step of this procedure, *E. coli* W3110 was transduced with  $\lambda::Tn10$  to generate a *Tn10* hop pool of *E. coli* W3110 [Kleckner *et al.*, J. Mol. Biol., *supra*].

The *E. coli* W3110::*Tn10* hop pool was grown in L broth at 37°C to a cell density of about  $1 \times 10^9$ /ml. A total of 0.5 ml of the culture was centrifuged and the pellet was resuspended in 0.2 ml of a  $\lambda$ phi80 lysate containing  $7.0 \times 10^8$  pfu. The phage was allowed to adsorb for 30 minutes at 37°C. The suspension was then spread on EMB plates supplemented with tetracycline (15  $\mu$ g/ml). After an overnight incubation at 37°C, the colonies were pooled in 3 ml of L broth, grown overnight at 37°C, washed twice, and resuspended in L broth. A bacteriophage P1kc lysate was made on this culture [Miller, J.H., Experiments in Molecular Biology, *supra*, page 304].

*E. coli* AT982 (no. 4546, *E. coli* Genetic Stock Center, New Haven, Conn.) was transduced to tetracycline resistance by this P1kc lysate. Transductants were selected on L broth plates supplemented with tetracycline (15  $\mu$ g/ml) and 40  $\mu$ g/ml diaminopimelic acid (dap). The resulting transductants were screened for tetracycline resistance and the regeneration of the *dap* gene (*dap*<sup>+</sup>, *ter*<sup>R</sup>). Transductants with the *dap*<sup>+</sup>, *ter*<sup>R</sup> genotype were then tested for  $\lambda$ phi80 resistance.

P1kc lysates were then made on several *dap*<sup>+</sup>, *ter*<sup>R</sup>,  $\lambda$ phi80-resistant strains. The lysates were used to transduce *E. coli* W3110 to tetracycline resistance. The transductants were screened and selected for  $\lambda$ phi80 resistance.

Tetracycline-sensitive isolates were selected from the W3110 *tonA::Tn10- $\lambda$ phi80R* transductants. [Maloy and Nunn, J. Bacteriol., **145**: 1110 (1981)]. These isolates were checked for  $\lambda$ phi80 resistance and tetracycline sensitivity after single colony purification.

DNA was isolated from several tetracycline-sensitive  $\lambda$ phi80-resistant mutants and digested with SstII. The SstII-digested DNA was characterized by the Southern blot procedure using radioactively labeled and SstII-digested  $\lambda::Tn10$  DNA as a probe to determine if the *Tn10* had been excised [Davis *et al.*, Advanced Bacterial Genetics (Cold Spring Harbor Laboratory, New York, 1980)]. One of the tetracycline-sensitive isolates was shown to have lost two of the *Tn10* hybridization bands as compared to the hybridization between DNA from



the  $\lambda::Tn10$  and the parental W3110  $tonA::Tn10\lambda phi80R$ . A third hybridization band had an altered mobility, indicating that a deletion caused by the imprecise excision of  $Tn10$  had occurred.

SDS-gel electrophoresis of outer membrane preparations from the strain with an imprecise  $Tn10$  excision revealed that the band assumed to be the protein encoded by  $tonA$  had an altered electrophoretic mobility as compared to the wild-type protein encoded by the  $tonA$  gene. The resulting protein was non-functional as a  $\lambda phi80$  phage receptor protein. A second independent strain that also had undergone imprecise excision of  $Tn10$  showed no protein encoded by  $tonA$  on the SDS gel.

Neither of these strains demonstrated reversion to tetracycline resistance or to  $\lambda phi80$  susceptibility, indicating that there was an imprecise excision of all or part of the  $Tn10$  transposon together with either a partial or complete deletion of the  $tonA$  gene. Thus, the protein encoded by the  $tonA$  gene (MW 78,000) was eliminated from the outer membrane, rendering the W3110  $tonA$  strain resistant to several bacteriophages. The resulting strain, designated 1A2, is resistant to bacteriophages T1 and  $\phi 80$ .

The  $ptr3$  gene [Cheng *et al.*, *J. Bacteriol.*, **140**: 125-130 (1979)] was introduced into strain 1A2 as follows. First, the  $thyA8$  mutation was isolated in 1A2 by selecting for trimethoprim resistance to form strain 9E1. Then the  $argA81::tn10$  locus was transported from 9D9 (obtained from B. Bachman, *E. coli* Genetic Stock Center, New Haven, Conn.) into 9E1 by transduction with phage P1kc to form 9E3. The  $ptr3$  locus is between  $thyA8$  and  $argA81$ . Transduction with P1 phage grown on a  $ptr3$  mutant [9D7, *J. Bact.*, **140**: 125 (1979)] resulted in the introduction of the  $ptr3$  mutation simultaneously with the conversion of  $thyA8$  and  $argA81::Tn10$  to wild-type loci. This strain, designated 9E4, lacks the periplasmic protease III. The conclusion that the  $ptr3$  mutation is included in 9E4 is supported by strongly improved IGF-I accumulation in the resultant strain.

Then, two more deletion mutations,  $phoA\Delta E15$  [Sarchy *et al.*, *J. Bacteriol.*, **145**: 288-292 (1981)] and  $\Delta(argF-lac)169$  [Schweizer and Boos, *Mol. Gen. Genet.*, **192**: 293-294 (1983)], were simultaneously transferred into 9E4 by genetic linkage to a kanamycin-resistance transposon inserted into a proline biosynthetic gene ( $proC::Tn5$ ), contained in 6D3, obtained from Professor Barry Wanner, Purdue University. This construction is diagrammed in Figure 2B.

The transposon was eliminated by selecting for a prototrophic ( $pro^+$ ) isolate on glucose minimal agar plates after P1 transduction with 1A2. The introduction of the  $phoA$  mutation eliminates alkaline phosphatase expression and was recognized as transductants that form white colonies on glucose-minimal agar plates with 0.2 mM phosphate and 20 mg/l 5-bromo-4-chloro-3-indolyl phosphate. Likewise, the  $\Delta(argF-lac)169$  mutation causes the loss of the enzyme beta-galactosidase (a  $lac^-$  phenotype) and results in cells that form white colonies on MacConkey-1% lactose agar plates. The resultant strain was designated 27A7.

The *ompT* deletion [Earhart *et al.*, FEBS Microbiol. Lett., 6: 277-280 (1979)] was introduced into 27A7 by P1 cotransduction. It should be noted that this *ompT* deletion extends into the neighboring *ent* gene cluster which codes for the attachment proteins for colicins B and D. First, a linked *Tn10* insertion in the *purE* gene was inserted next to the *ompT* deletion using conventional transduction techniques [intermediates 3E9 (similar strain obtainable from Dr. Carol Gros, University of Wisconsin), 16B2, 25C9 (J. Bacter., 153: 1104-1106 (1983)), and 25D3]. Then, the *purE::Tn10* was transduced into 27A7. Finally, this strain was transduced to purine prototrophy to remove the transposon. Maintenance of the *ompTΔ* genotype was confirmed by colicin B resistance in the resultant strain, which is designated 27C6. This strain lacks the outer membrane protease VII.

Finally, an additional periplasmic protease mutation, *degP41kan<sup>r</sup>* [Strauch *et al.*, J. Bacteriol., 171: 2689-2696 (1989); Harvard Medical School] was transduced into strain 27C6 by standard techniques. This mutation was constructed *in vitro* by replacing a section of the *degP* gene with the kanamycin gene. This is not a transposon but allows for selection of the deletion using kanamycin resistance.

This final strain, 27C7, has the following characteristics: it is phage resistant, lacks three proteases, fails to grow on lactose, and fails to produce alkaline phosphatase on the depletion of phosphate in the media, the same conditions that induce production of rhIGF-I.

#### ii. Description/Construction of Expression Plasmid pLS32Tsc

The secretion plasmid pLS32Tsc used to transform strain 27C7 contains the IGF-I gene. The transcriptional and translational sequences required for expression of the IGF-I gene in *E. coli* are provided by the alkaline phosphatase promoter and the *trp* Shine-Dalgarno sequence. The lambda *t<sub>o</sub>* transcriptional terminator is situated adjacent to the IGF-I termination codon. Secretion of the protein from the cytoplasm is directed by the *lamB* signal sequence or alternatively the STII signal sequence. The majority of rhIGF-I is found in the cell periplasmic space. Plasmid pLS32Tsc confers tetracycline resistance upon the transformed host.

Plasmid pLS32Tsc was constructed in several steps using as intermediate plasmids pLS32, pAPlamB, pLS32lamB, pLS33lamB, and pLS33Tsc.

#### 30 Step 1: pLS32

The plasmid pLS32 results in the fusion of the IGF-I coding sequence to that of the heat-stable enterotoxin II (STII) signal sequence and was prepared by ligating together four DNA fragments as shown in Figure 4. The first of these was the vector pTF2A12 [Paborsky *et al.*, Biochemistry, 28: 8072-8077 (1989)] from which the small *NsiI*-*Bam*HI fragment containing the tissue factor gene had been removed. The STII signal sequence is described by Picken *et al.*, Infect. Immun., 42: 269-275 (1983).

The second fragment was a 55-bp synthetic duplex encoding the first 18 amino acids of mature IGF-I. This duplex has the following sequence:

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5' - GGTCCCGAAACTCTGTGCGGTGCTGAACTGGTTGACGCTCTGCAGTTTGTTC-  
 3' - CGTCCAGGGCTTTGAGACACGCCACGACTTGACCAACTGCGAGACGTCAAACAAACG-

G-3'  
 CCACTG-5'

(Sequence Identity Nos. 1 and 2, respectively)

The third piece in the ligation was a 154-bp BstEII-HindIII fragment from pK1ZZIGF-I encoding the remaining amino acids 19-70 of IGF-I. pK1ZZIGF-I is a kanamycin-resistant plasmid containing a lac promoter attached to a Protein A promoter attached to a Protein A signal, attached to two consensus Z regions from Protein A that bind IgGs and secrete proteins, fused using two codons encoding an Asn-Gly interface to a synthetic IGF-I gene and also containing an F region to give high copy number. This plasmid is similar to pZZ-IGF-I shown in Fig. 6 of and described in EP Pub. No. 230,869 published 5 August 1987, where the ampicillin gene is replaced by a kanamycin gene.

The last fragment was a 291-bp HindIII-BamHI fragment from the plasmid pLS8. This last fragment is simply the coding sequence for the start of the tetracycline gene of pBR322 [Sutcliffe, Cold Spring Harbor Symposia on Quantitative Biology, 43: 77-90 (1978)] in which a HindIII restriction site was engineered immediately upstream of the methionine start codon.

The resulting plasmid, pLS32, efficiently expresses and secretes rhIGF-I to the media. The following two construction steps were made to replace the STII signal sequence with the lamB signal sequence, improving product yield.

#### Step 2: pAPlamB

The plasmid pAPlamB was constructed as shown in Figure 5 by ligating together two DNA fragments, and results in the placement of the lamB signal coding sequence downstream of the AP promoter and the trp Shine-Dalgarno sequence. Included in the ligation was the vector pRA1 in which the small XbaI-BglII fragment had been removed. This plasmid is a derivative of pHGH1 [Chang *et al.*, Gene, 55: 189-196 (1987)], which latter plasmid contains the AP promoter, the STII signal, and DNA encoding hGH. pRA1 differs from pHGH1 in that it contains DNA encoding relaxin A chain (the sequence of which is described in U.S. Pat. No. 4,758,516) rather than hGH and it contains a convenient BglII restriction site downstream of the promoter and ribosome binding site. The second piece in the ligation was a 80-bp synthetic DNA duplex with the following sequence, which encodes the lamB signal sequence, which has been described by Clement and Hofnung, Cell, 27: 507-514 (1981):

5' - CTAGAATTATGATGATTACTCTGCGCAAACCTTCTCTGGCGGTTGCCGTCGCAGC-  
 3' - TTAATACTACTAATGAGACGCGTTTGAAGGAGACCGCCAACGGCAGCGTCG-  
 GGGCGTAATGTCTGCTCAGGCCATGGCCA-3'  
 CCCGCATTACAGACGAGTCCGGTACCGGTCTAG-5'

(Sequence Identity Nos. 3 and 4, respectively)

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Step 3: pLS32lamB

The plasmid pLS32lamB results in the fusion of the lamB signal sequence to the IGF-I coding region and was constructed as shown in Figure 6 by the ligation of three DNA fragments. The first of these was the vector pLS32 in which the small XbaI-BstEII fragment had been removed. The second was a 75-bp XbaI-EaeI fragment from pAPlamB encoding the lamB signal sequence. The third was a 55-bp synthetic DNA duplex encoding the first 18 amino acids of mature IGF-I, and having the following sequence:

5' - GGCCGGTCCCGAAACTCTGTGCGGTGCTGAACTGGTTGACGCTCTGCAGTTTGT -  
 3' - CCAGGGCTTTGAGACACGCCACGACTTGACCAACTGCGAGACGTCAAACA -  
 TTGCG - 3'  
 AACGCCACTG - 5'

(Sequence Identity Nos. 5 and 6, respectively)

The following steps introduce into the plasmid the transcriptional terminator. These plasmid changes resulted in an improved product yield.

Step 4: pLS33lamB

The plasmid pLS33lamB is an intermediate in the preparation of pLS32Tsc and was constructed as shown in Figure 7 by ligating together three DNA fragments. The first of these was the vector pLS32 in which the small XbaI-BstEII fragment had been removed. The second was a 75-bp XbaI-EaeI fragment from pAPlamB encoding the lamB signal sequence. The third was a 46-bp synthetic DNA duplex with the following sequence:

5' - GGCCACTCTGTGCGGTGCTGAACTGGTTGACGCTCTGCAGTTTGTGCG - 3'  
 3' - TGAGACACGCCACGACTTGACCAACTGCGAGACGTCAAACAAACGC -  
 CACTG - 5'

(Sequence Identity Nos. 7 and 8, respectively)

The above sequence encodes amino acids 4-18 of mature IGF-I.

Step 5: pLS33Tsc

The plasmid pLS33Tsc results in the placement of the lambda  $t_{\text{c}}$  transcriptional terminator immediately downstream of the IGF-I coding sequence. Three DNA fragments were ligated together as shown in Figure 8 to construct this plasmid. The first piece was the vector pLS18 in which the small XbaI-BamHI fragment had been removed. pLS18 is a derivative of pHGH1 [Chang *et al.*, *supra*] that contains DNA encoding human DNase (as disclosed in WO 90/07572 published July 12, 1990) rather than hGH. pHGH1 could be used to generate the same fragment. The second part of the ligation was a 288-bp XbaI-HindIII fragment from pLS33lamB in which the HindIII restriction site had been blunted by treatment with DNA polymerase I (Klenow). The third part of the ligation was a 412-bp StuI-BamHI fragment from the plasmid pdH108-4. This fragment contains the lambda  $t_{\text{c}}$  transcriptional terminator [Scholtissek and Grosse, *Nuc. Acids Res.*, **15**: 3185 (1987)] and base pairs 2-375 of pBR322 [Sutcliffe, *supra*], wherein the base pairs 2-375 are downstream or 3' of the

transcriptional terminator. The sequence of the terminator region of this fragment is as follows:

5' - CCTAACGCTCGGTTGCCGCCGGGCGTTTTTATTGTTAA - 3'  
3' - GGATTGCGAGCCAACGGCGGCCCGCAAAAAATAACAATT - 5'

5 (Sequence Identity Nos. 9 and 10, respectively)

Step 6: pLS32Tsc

10 The final plasmid pLS32Tsc was constructed as shown in Figure 9 by ligating together two DNA fragments. The first of these was the vector pLS33Tsc from which the small EcoRI-BstEII fragment had been removed. The second was a 550-bp EcoRI-BstEII fragment from pLS32lamB containing the AP promoter, trp Shine-Dalgarno, and the coding sequence for the lamB signal sequence fused to the first 18 amino acids of IGF-I. The resulting plasmid was analyzed by restriction endonuclease digestion. The entire promoter and coding sequence was verified by DNA sequencing, with the sequence being given in Figure 10 (Sequence Identity No. 11).

15 iii. Fermentation and Recovery Procedure

Competent *E. coli* 27C7 cells were transformed with pLS32Tsc by standard transformation techniques. Transformants were selected and purified on LB plates containing 20 mg/L tetracycline. This medium had the following composition: 10 g/L Bacto-Tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, and 20 mg/L tetracycline-HCl.

20 One transformed colony was used to inoculate sterile LB broth containing 20 mg/L tetracycline. The flask culture was incubated at 35-39°C until the optical density at 550 nm reached approximately 1.0. Sterile DMSO was added to the culture to give a final concentration of DMSO of 10% (v/v). Aliquots of 1-2 mL were dispensed into sterile vials and stored at -60°C or below.

25 The fermentation process for producing rhIGF-I using 27C7/pLS32Tsc was performed in batches ranging in volume from 5 to 12 liters. At the end of the fermentation, cells were harvested by centrifugation.

30 A shake flask inoculum was prepared by inoculating approximately 500 ml of sterile LB medium containing tetracycline with the freshly thawed 1-2 ml culture vial described above. The shake flask was incubated at 35-39°C at 50-200 rpm for 7-12 hours. The shake flask was then used to inoculate a 15-liter fermentation vessel containing 5-9 liters of culture medium composed as follows:

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	<u>Ingredient</u>	<u>Quantity/Liter</u>
	glucose*	250-350 g
	ammonium sulfate	2-6 g
5	ammonium hydroxide	as required to control pH 7.1 to 7.5
	sodium phosphate, monobasic dihydrate	1-2 g
	potassium phosphate, dibasic	2-3 g
	sodium citrate, dihydrate	0.5-1.5 g
10	potassium chloride	1-2 g
	25% Pluronic Polyol L61	0.2 ml initially and as needed to control foaming
	magnesium sulfate, heptahydrate	1-3 g
15	tetracycline HCl	5-20 mg
	yeast extract**	5-15 g
	NZ amine AS**	5-20 g
	isoleucine	0-10 g
20	ferric chloride, heptahydrate	10-30 mg
	zinc sulfate, heptahydrate	2-5 mg
	cobalt chloride, hexahydrate	2-5 mg
	sodium molybdate, dihydrate	2-5 mg
	cupric sulfate, pentahydrate	2-5 mg
25	boric acid	0.5-2 mg
	manganese sulfate, monohydrate	1-3 mg
30	* 2-5 g/L of glucose was added to the culture initially. The remainder was fed to the culture over the course of the fermentation at rates rapid enough to allow rapid growth during the initial part of the fermentation, but not so rapid as to cause the dissolved oxygen level to fall below 30% of air saturation levels during the latter portion of the fermentation (when significant cell mass has accumulated).	
35	** Yeast extract and NZ amine AS can be added initially and/or fed throughout the fermentation.	
40	The fermentation process was performed at 35-39°C at pH 7.1-7.5 for 24-48 hours. The agitation rate was set at 650-1000 rpm and the aeration rate at 0.7-1.5 volumes of air per volume of culture per minute. Production of IGF-I occurred after the phosphate in the medium was depleted. After the fermentation, the culture was chilled and then harvested by centrifugation. Harvested cells were resuspended in cell lysis buffer (approximately 4 g cell paste/100 ml) containing 25 mM Tris, pH 7.5, 5 mM EDTA, with 200 µg/ml hen egg white lysozyme, and sonicated at 4°C for approximately 5 min. Cell lysates were centrifuged at 5,000 to 15,000 x g at 4°C for 10 min and the supernatant and pellet fractions were	

analyzed on reduced and non-reduced SDS gels.

iv. Results

A. Distribution of IGF-I

The reduced gel revealed that for whole cell lysates, approximately 10% of total cell protein is IGF-I, contained almost exclusively in the pellet fraction. On the non-reduced gel, the IGF-I band was nearly absent while numerous high molecular weight faint bands appeared, suggesting that the majority of cell-associated IGF-I is in a disulfide-linked, aggregated form.

B. Solubilization of IGF-I in Tris buffer

The refractile body material from a 4 ml OD cell pellet, isolated as described above, was solubilized in 100  $\mu$ l of 25 mM Tris, pH 7.5, plus 5 mM EDTA and varying amounts of DTT and chaotropic agent (urea or guanidine chloride). Solubilization of the refractile particles was examined by observing the clearing of the refractile particle suspensions (i.e., the decrease in turbidity of the suspension) and was confirmed by centrifuging the samples and conducting Coomassie blue-stained PAGE gel analysis of the resulting supernatant and pellet fractions. The protein concentration in the supernatant (also an indication of solubility) was measured by the protein assay kit provided by BioRad (Richmond, CA). Supernatant was also assayed for IGF-I by radioimmunoassay (RIA). Refractile body protein was not soluble with either chaotropic agent alone or DTT alone; however, in combination, the protein was efficiently solubilized. See Table 1.

TABLE 1

Solubilization with Minimal Chaotropic Agent  
and DTT in Tris Buffer

	<u>Chaotropic Agent (M)</u>	<u>DTT (mM)</u>	<u>Solubility*</u>	<u>IGF-I by RIA (<math>\mu</math>g/ml OD)</u>
	0 agent	0	-	0.05
30	0 urea	10	-	0.00**
	2 urea	"	+	0.47**
	4 urea	"	++++	1.09**
	6 urea	"	++++	1.10**
	8 urea	"	++++	1.11**
35	4 urea	1	+++	0.53**
	6 urea	"	+++	1.81**
	8 urea	"	+++	0.69**
	2 urea	"	+++	0.72**
	" urea	2	+++	0.10**
40	" urea	3	+++	0.08**
	" urea	4	++++	0.04**
	4 urea	0	-	0.06
	" urea	1	+++	0.57
	" urea	2	++++	0.08
45	" urea	4	++++	0.06
	" urea	6	++++	0.08

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	" urea	8	++++	0.15
	" urea	10	++++	0.13
	3 urea	10	+++	0.19
	2 urea	0	-	0.06
5	4 urea	0	-	0.08
	6 urea	0	-	0.10
	8 urea	0	-	0.11
	8 urea	10	++++	0.05
	2 GuCl***	0	-	0.14
10	4 GuCl	0	-	0.12
	6 GuCl	0	-	0.10
	6 GuCl	10	++++	0.08

15

\* "-" designates not soluble; "++++" is the most soluble.

\*\* Refolded for five hours; no "\*\*\*" designates refolded for two hours.

20

\*\*\*GuCl designates guanidine hydrochloride.

25

Apparently, complete solubilization was attained with 2 M urea/4 mM DTT or at least 4 M urea and at least 2 mM DTT. It was found that the RP-HPLC peak area corresponding to the correct, authentic form of IGF-I accounts for all of the RIA titer, suggesting that when soluble IGF-I becomes RIA recognizable, it acquires a correctly folded conformation. Only minor amounts of IGF-I were detectable by RIA, even in the completely solubilized protein samples, indicating that the tertiary structure of the protein solubilized under these conditions was not the same as that of authentic IGF-I.

30

At pH 7.5 or less, Tris buffer does not result in significant yield of correctly folded IGF-I; however, correct refolding occurs in Tris buffer above pH 8.0. See Fig. 11A, where various buffers are compared, described more fully in Example III, Section C.

## EXAMPLE II

35

### Test for Solubility of IGF-I in Acetate Buffer and for Refolding and Effect of Incubation Temperature

#### A. Solubilization and refolding of IGF-I in sodium acetate buffer

Cell lysates were prepared as above through the cell harvesting step. Refractile body protein was solubilized in 100 mM sodium acetate (NaOAc) pH 8.2 containing 100 mM NaCl and in varying concentrations of urea and DTT.

40

At least 70% of the refractile body protein was soluble with 2 M urea and DTT at 2 mM or greater. Optimal solubility and IGF-I titer was obtained with 2 M urea, 2-4 mM DTT. See Table 2. In the absence of DTT, both solubility and IGF-I titer were insignificant.



TABLE 2

Effect of Urea and DTT Concentrations at pH 8.2

	<u>Urea</u> <u>(M)</u>	<u>DTT</u> <u>(mM)</u>	<u>Solubility</u>	<u>IGF-I by RIA</u> <u>(<math>\mu</math>g/ml OD)</u>	<u>Protein</u> <u>(mg/ml)</u>	<u>%</u> <u>Refold</u>
5	0	0	-	0.01		
	1	10	++	0.05		
	2	0	-	0.12		
	"	1	++	4.76		
10	"	2	+++	5.43		
	"	4	++++(+)	5.85		
	"	6	++++	2.05		
	"	8	++++	0.88		
	"	10	++++	0.27		
15	1	0	-	0.08		
	"	1	++	4.61		
	"	2	+++	3.74		
	"	4	+++	0.52		
20	2	0	-	0.12	0.17	3
	"	1	++	5.82	0.62	37
	"	2	++++	6.88	1.22	22
	"	4	++++	6.23	1.35	18
	3	0	-	0.11		
25	"	1	++	4.81		
	"	2	+++	5.02		
	"	4	+++	4.00		
	4	0	-	0.04		
30	"	1	-(+)	0.15		
	"	2	+	0.26		
	"	4	+++	0.13		

In the presence of 1 or 2 mM DTT and 2 M urea, varying the starting cell concentration (used to obtain refractile body protein) from 0.10 to 4.00 ml-OD indicated that the higher concentrations tested were more favorable for refolding only when 2 mM DTT was employed. See Table 3. Protein from 1-4 ml-OD of cell pellet was optimal.

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TABLE 3

Effect of IGF-I Concentration on Refolding

	DTT (mM)	Starting	Solubility	RIA ( $\mu$ g/ml-OD)	Soluble Protein		% Refold
		Cells (ml-OD)			Concentration (mg/ml)    ( $\mu$ g/mlOD)		
5	1	0.10	+++	9.4	0.07	71	13
	"	0.25	+++	11.8	0.22	86	14
	"	0.50	+++	15.2	0.33	65	46
10	"	1.00	+++	8.9	0.33	33	27
	"	4.00	++	9.2	1.08	27	34
2	0.10		++++	0.2	0.13	130	0.2
	"	0.25	++++	0.1	0.30	121	0
	"	0.50	++++	1.9	0.33	67	2.3
15	"	1.00	++++	11.8	0.52	52	23
	"	4.00	+++	8.2	1.35	34	24

In a separate determination of the effect of IGF-I concentration on folding (with constant IGF-I/DTT), the results shown in Figure 12 were obtained, indicating an optimum IGF-I concentration for optimum yield corresponding to 1.5 to 5 mg/ml (approximately 0.2 to 0.7 mM).

#### B. Altering incubation temperature

Refractile body protein was obtained as described above and solubilized in acetate buffer containing 2 M urea and 2 mM DTT. Refolding was allowed to proceed 4 hours at various temperatures. Refolding at 4°C reduced RIA titers 50% compared to 23°C or 37°C.

See Table 4.

TABLE 4

Effect of Temperature

	Temperature (°C)	Solubility	IGF-I RIA ( $\mu$ g/ml OD)
30	4	++++	4.60
	23	++++	9.14
	37	++++	8.66

In a separate experiment where the folding was done in CAPSO buffer, pH 10.5 in the presence of 20% methanol, the amount of correct IGF-I obtained was optimized between 15 and 37°C. See Table 5.

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TABLE 5

Effect of Temperature

	<u>Temperature</u> <u>(°C)</u>	<u>Correct IGF-I</u> <u>(<math>\mu</math>g/OD ml)</u>	<u>Correct/</u> <u>Misfolded</u>	<u>Methionyl</u> <u>Sulfoxide</u> (%)
5	4	10.6	2.5	2.6
	15	17.8	3.7	2.2
10	23	18.2	3.9	2.1
	37	15.3	5.1	3.1

\* % of methionyl sulfoxide as compared to correct IGF-I level.

15

C. Altering incubation time

When the temperature was kept at 23°C and the time interval varied, with pH 8.2, using intervals of 1/2, 1, 2, 3, 4, 5, and 24 hours, RIA titers increased with time, reaching a plateau at about 5 hours. See Fig. 13A.

20

In addition, refolding was carried out using CAPSO buffer at pH 10.5 with and without methanol. The data indicated that IGF-I titers (by HPLC assay) increased with time and plateaued. See Figs. 13B (no methanol) and 13C (with methanol).

D. HPLC profile of refolded refractile body protein

25

Refolded protein, solubilized as described in Section B above at 23°C for 4 hours, was examined by HPLC on a reverse-phase Vydac C-18 column at pH 2.0. Two major IGF-I peaks eluted, one co-migrating with authentic IGF-I and the other with a misfolded form in a ratio of 1.5/1 to 5/1. Refractile bodies solubilized in 2 M urea and 1-4 mM DTT yield 25-30% of properly folded IGF-I in acetate buffer as determined from the RIA titers, which are a measure of the amount of authentic IGF-I present.

30

## EXAMPLE III

## Effects of Various Parameters on Refolding

A. Characterization of refolded IGF-I

35

Cell lysates were prepared as above through the cell harvesting step. Refractile body protein from a 200 ml-OD cell paste was solubilized in 10 ml of buffer comprising 100 mM NaOAc, pH 8.2, 100 mM NaCl, 2 M urea, and 2 mM DTT. Refolding of refractile body protein proceeded 4 hrs at 23°C. The protein was evaluated using preparative reverse-phase HPLC at pH 7.0 on a Waters-C4 column, at pH 7.0. Fractions which co-migrated with authentic IGF-I were subjected to V-8 protease digestion and mass spectrometry analysis.

40

RIA of the refolded protein revealed an IGF-I titer of 7.9  $\mu$ g/ml OD. The protein pattern of the refolded sample was compared to that of the refractile body protein prior to refolding, using Coomassie blue-stained 10-20% Tricine gels. Under reduced gel conditions, only one band was visible corresponding to the IGF-I monomer migrating near 6.2 Kd. Under non-

reduced gel conditions, bands corresponding to monomer, dimer, and oligomer forms were detected for the refolded but not for the non-refolded sample. Under non-reduced gel conditions, the non-refolded sample contained numerous high molecular weight bands but no band migrating near the 6.2 Kd monomer.

5 After preparative reverse phase HPLC of the refolded sample, two major IGF-I peaks were obtained. One co-migrated with authentic IGF-I and the other co-migrated with misfolded IGF-I. A significant amount of protein was also found in a regenerate fraction, containing aggregates of IGF-I. When the protein pattern of each of these two major peak fractions was displayed on SDS-Tricine reduced gels, each contained only one band which  
10 co-migrated with IGF-I. The distribution of IGF-I, as adduced by the intensity of the stained bands, was approximately 35/25/40 in correctly folded:misfolded:regenerate forms. In non-reduced gels, more than 50% of IGF-I in the regenerate fraction appeared as dimers and oligomers. The results indicate that the correctly folded and misfolded IGF-I exhibit identical mobility in reduced and non-reduced gels and the majority of IGF-I in the regenerate fraction  
15 is in a disulfide-linked oligomeric form.

The fractions recovered after HPLC were also analyzed by radioimmunoassay for IGF-I and by bioassay for IGF-I activity. The bioassay measures the ability of the IGF-I to enhance the incorporation of tritiated thymidine, in a dose-dependent manner, into the DNA of BALB/c 3T3 fibroblasts, performed, with some modifications, according to the procedure described  
20 by Tamura *et al.*, J. Biol. Chem., **262**: 5616-5621 (1989). High IGF-I RIA titers were found only in the fraction co-migrating with correctly folded IGF-I. Similarly, IGF-I activity was high only in that same fraction. See Table 6.

TABLE 6  
IGF-I Titers of Various Fractions from HPLC

25	Fractions	Co-migrate with	IGF-I titers( $\mu$ g/ml OD)	
			RIA	Bioassay
	2-13		ND*	ND
	13-28		ND	ND
30	28-32		ND	ND
	32-38		0.04	ND
	38-46		0.12	ND
	46-51	authentic IGF-I	2.40	5.46
	51-55	misfolded IGF-I	0.13	0.16
35	55-58	regenerate	0.29	1.72

\* ND is not detectable.

To characterize the disulfide linkage and check the amino acid sequence of refolded protein, the HPLC fractions 46-51, co-migrating with authentic IGF-I, were subjected to V-8 protease digestion and then analyzed by HPLC. The profile is almost identical to that of

authentic IGF-I. Several small variant peaks, analyzed further by mass spectroscopy, were identified as incomplete digests of IGF-I fragments. These results indicate that the refolded protein co-migrating with correctly refolded material contained correct disulfide linkages and correct terminal sequences. The refolded protein co-migrating with misfolded protein was similarly analyzed. The results indicated that two of the three disulfide bonds were formed incorrectly.

#### B. Effect of pH on refolding

Refractile body protein from 4 ml-OD cell pellet was resuspended in 100  $\mu$ l of NaOAc buffer ranging in pH from 4.1-8.2, all containing 100 mM NaCl, 2 M urea, and 2 mM DTT. Solubilization and refolding proceeded for 5 hrs at 23°C. See Table 7.

TABLE 7

Effect of pH on IGF-I Refolding

pH	Solubility	IGF-I ( $\mu$ g/ml OD)
4.1	-	0.02
4.4	-	0.03
5.0	-	0.04
6.0	+	0.08
6.4	++	0.06
8.2	+++	5.18

Turbidity measurements revealed that the protein was completely insoluble below pH 6.0 and became almost completely soluble at pH 8.2. This was confirmed by soluble protein measurement and PAGE. RIA for IGF-I revealed that correct refolding occurred in a significant amount at pH 8.2 but not at the lower pH values tested. C. Effect of buffer and pH on refolding

Various buffers were examined at a range of pH within the effective range of each buffer. These buffers and their pKa are: glycylglycine, pKa 8.4, Taps, pKa 8.4, Tris, pKa 8.3, Bicine, pKa 8.3, Tricine, pKa 8.1, Hepes, pKa 7.5, Ampso, pKa 9.0, Ches, pKa 9.3, Capso, pKa 9.6, Amp, pKa 9.7, Caps, pKa 10.4. All buffers contained 100 mM NaCl, 2 M urea, and 2 mM DTT. Refractile body protein from 4 ml-OD cell pellet was resuspended in 100  $\mu$ l of each buffer. Refolding proceeded for 5 hrs at 23°C.

Turbidity data, soluble protein measurements, and PAGE indicated that among buffers with slightly basic pKa (pKa 7.5-8.4), solubility of IGF-I increased as the pH increased. At pH 9 and above, almost all IGF-I was solubilized. For Tris, Taps, Bicine, and Glycylglycine buffers, correct refolding, as measured by RIA for IGF-I, increased significantly as pH increased from about 7.5 to 9.5. See Fig. 11A.

Among buffers with very basic pKa (pKa  $\geq$  9.0) IGF-I was almost completely soluble within the useful pH range of these buffers (pH 8.5-11). RIA for IGF-I revealed high titers in

all these buffers, for the entire pH range tested. See Fig. 11B. Figures 13A-C show a typical time sequence for these reactions.

#### EXAMPLE IV

##### Refolding of IGF-I Produced By Various *E. coli* Hosts

5

10

Three *E. coli* periplasmic and membrane protease deficient hosts, designated 27A7, 27B4, and 27C7, carrying the IGF-I secretion plasmid pLS32Tsc (described above), were used as a source of refractile body protein. The construction of strains 27A7 and 27C7 is described in Example I. Strain 27B4, which has the complete genotype W3110 *tonAΔ ptr3 phoAΔE15 Δ(largF-lac)169 degP41*, was prepared by transducing the *degP41* periplasmic protease mutation [Strauch *et al.*, *supra*] into strain 27A7. This mutation was constructed *in vitro* by replacing a section of the *degP* gene with the kanamycin gene to allow for selection of the deletion using kanamycin resistance.

15

Refractile body protein was isolated from each of these three host strains as previously described from 0.2 g of cell paste in a 5 ml volume. Refractile body protein from a 4 ml-OD cell pellet was resuspended in 100  $\mu$ l of buffer comprising 100 mM CAPSO, pH 10.5, 100 mM NaCl, 2 M urea, and 1-4 mM DTT. Refolding proceeded 5 hrs at 23°C.

20

During isolation of the refractile body protein, samples were taken to identify the protein distribution from the various hosts. The protein patterns were displayed on Coomassie blue stained reduced SDS gels. For whole cell lysates, approximately 5% and 10% of total protein was IGF-I for the strain 27A7, and for the 27B4 and 27C7 strains, respectively. Supernatant fractions of cell lysates exhibited protein patterns similar to the cell lysates but with a very faint IGF-I band. Pellet fractions (refractile bodies) exhibited IGF-I banding almost exclusively.

25

30

IGF-I refolding was measured by RIA. Only the pellet fractions exhibiting an IGF-I band showed significant IGF-I titer in the RIA. The IGF-I titers were highest in the fractions with the darkest staining IGF-I bands, which provides confirmatory data for higher IGF-I concentrations in these fractions. The various *E. coli* hosts and expression levels did not affect the extractability, purity, or refolding of the refractile body proteins. Refractile body proteins from all strains refolded with similar relative yields, as determined from the observation that IGF-I titers correlated well with the intensity of the IGF-I band on the SDS gels.

#### EXAMPLE V

##### Measurement of Total IGF-I (correct + misfolded) From Host Cells

35

Triple-protease deficient *E. coli* cells, as described in Example 1 (27C7), were extracted in 50 mM Tris buffer, pH 8.0, 6 M urea, 5 mM EDTA, 10 mM DTT. Following centrifugation, the supernatant fraction was eluted through two PLRP-S columns (Polymer

Labs Reverse Phase-S) with a linear gradient of 32-45% acetonitrile combined with step gradients. The IGF-I eluted in a peak well separated from those of *E. coli* proteins. The levels of cell-associated and refractile body IGF-I were calculated from peak data as 4.5 g/l and 3.8 g/l respectively. This indicated there is 85% recovery of IGF-I in the refractile body protein preparation.

Cell-associated IGF-I is calculated as 4.7 g/l for a 10 liter fermentation at 100 OD. The IGF-I concentration is approximately 1.5 mg/ml in refractile body protein from a 4 OD-ml *E. coli* cell pellet in 100  $\mu$ l refolding buffer.

#### EXAMPLE VI

##### Effect of Hydrophobic Agents on IGF-I Refolding

Refractile body protein from a 4 OD-ml *E. coli* strain 27C7 cell pellet was refolded in 100  $\mu$ l of IGF-I refolding buffer consisting of 100 mM CAPSO, pH 10.5, 100 mM NaCl, 2 M urea, 2 mM DTT and also containing either 20% ethanol or 20% methanol as hydrophobic agent. See Table 8.

TABLE 8

##### Effect of Hydrophobic Agents on IGF-I Refolding

<u>Agent</u> <u>(20% v/v)</u>	<u>Correct</u> <u>(<math>\mu</math>g/OD ml)</u>	<u>Misfold</u> <u>(<math>\mu</math>g/OD ml)</u>	<u>Correct/</u> <u>Misfolded</u>	<u>Increased</u> <u>Yield (%)</u> *
<u>Run 1</u>				
-	12.2	5.1	2.4	-
Methanol	17.2	4.2	4.1	20
<u>Run 2</u>				
-	14.2	5.6	2.5	-
Methanol	18.9	4.4	4.3	34
Ethanol	18.2	3.3	5.5	28

\*Enhanced by the organic solvent methanol or ethanol.

Refolding proceeded at 23°C for 5 hours. Addition of methanol or ethanol increased the ratio of correctly folded to misfolded IGF-I and the total yield (correct + misfolded), measured by peak analysis after HPLC with a Vydac C-18 column. The ratio of correct:misfolded was about 4.1-4.4 and 2.4-2.7 for samples with and without methanol, respectively. The ratios of several more hydrophobic minor IGF-I peaks also changed in the more hydrophobic buffer. It was also discovered that as the percent methanol increases from 0% up to 20%, the ratio of correct:misfolded increases.

## EXAMPLE VII

Comparison of  $\beta$ -mercaptoethanol (BME) and DTT  
as Reducing Agents

5        Refractile body protein from a 4 OD-ml *E. coli* cell pellet, as described in Example 1,  
was refolded in 100  $\mu$ l of IGF-I refolding buffer consisting of 100 mM CAPSO, pH 10.5, 100  
mM NaCl, 2 M urea, and varying concentrations of BME or DTT. Refolding proceeded at  
23°C for 5 hours. The ratio of correct to misfolded conformer and the yield was analyzed  
10        by HPLC of the solubilized protein through a Vydac C-18 column. See Table 9. The yield of  
total IGF-I (both correct and misfolded) was decreased with BME as reducing agent as  
compared to DTT as reducing agent at all concentrations. However, the ratio of correct to  
misfolded conformer was similar with either reagent.

TABLE 9

Effect of BME and DTT on IGF-I Refolding

15 <u>Reducing</u> <u>Reagent</u>	<u>Correct IGF-I</u> <u>(<math>\mu</math>g/OD ml)</u>	<u>Misfolded IGF-I</u> <u>(<math>\mu</math>g/OD ml)</u>	<u>Ratio</u> <u>Correct/</u> <u>Misfolded</u>
DTT, 1 mM	10.1	4.9	2.1
20        DTT, 2 mM	13.7	5.9	2.3
DTT, 4 mM	12.1	5.4	2.3
BME, 1 mM	8.5	3.2	2.7
BME, 2 mM	7.5	3.1	2.5
BME, 4 mM	6.2	2.8	2.3

## EXAMPLE VIII

Comparison of BME and DTT as Reducing Agents  
in the Presence of Methanol

25        Refractile body protein from a 4 OD-ml *E. coli* cell pellet, as described in Example 1,  
30        was refolded in 100  $\mu$ l of IGF-I refolding buffer consisting of 100 mM CAPSO, pH 10.5, 100  
mM NaCl, 2 M urea, 20% methanol, and varying concentrations of BME or DTT as reducing  
agent. Refolding proceeded 5 hrs at 23°C. The refolded protein was analyzed using a Vydac  
C-18 HPLC column to separate correct and misfolded forms. At 1 mM, 2 mM, or 4 mM DTT,  
HPLC of the refolded sample revealed a ratio of correct:misfolded IGF-I of about 4:1. Total  
35        yield (correct + misfolded) remained approximately constant regardless of concentration of  
DTT. See Fig. 14A.

BME was effective in promoting refolding at as low as 0.2 mM concentration. At  
concentrations above 0.8 mM BME, IGF-I was completely soluble. Three peaks eluted by  
HPLC were identified as the correct, misfolded, and methionyl sulfoxide conformers. IGF-I  
40        refolding was dependent on the concentration of BME, with maximum yield of IGF-I at 1-2  
mM BME. The yield is slightly lower (13%) than the maximum yield with DTT. The



correct:misfolded ratio was about 3:1 at low BME (0.2 mM) and increased to about 4.2:1 as BME concentration increased to 2 mM. However, levels of the methionine sulfoxide variants also increased with increasing BME concentration. See Fig. 14B.

#### EXAMPLE IX

##### Effect of varying IGF-I and DTT Concentrations

Refractile body protein from a 4 OD-ml *E. coli* cell paste, as described in Example 1, was refolded in 100  $\mu$ l of IGF-I refolding buffer consisting of 100 mM CAPSO, pH 10.5, 100 mM NaCl, 2 M urea, 20% methanol, and varying concentrations of DTT. Refolding proceeded 5 hrs at 23°C. The soluble, refolded protein was analyzed using a Vydac C-18 HPLC column to separate correct and misfolded forms. Insoluble protein was solubilized in 100  $\mu$ l of solution containing 6 M urea, 50 mM Tris, pH 8.0, 5 mM EDTA, and 10 mM DTT and analyzed using two PLRP-S columns connected in series. Solubilized protein in the refolding buffer was determined as the difference between total IGF-I protein in the refolding buffer and IGF-I in the pellet, calculated from the PLRP-S analysis. Total IGF-I from refractile body particles from 4 ml-OD cell paste is about 1.5 mg/ml.

Effects of varying concentrations of DTT and IGF-I refractile body (RB) protein are shown in Table 10 and in Figures 15A, 15B, 15C, and 15D, with Fig. 15A showing only correct IGF-I titer for either a single DTT concentration or a constant IGF-I/DTT ratio of 0.11. The other figures show IGF-I titers and ratios of correct to misfolded IGF-I at either a constant IGF-I/DTT ratio (Fig. 15B) or a constant DTT concentration (1 mM for Fig. 15D and 2 mM for Fig. 15C).

TABLE 10

Effect of Refractile Body Particle Concentration on IGF-I Refolding

	<u>RB (ODml/ 100A)</u>	<u>DTT (mM)</u>	<u>IGF/ DTT</u>	<u>Sol. IGF (mM)</u>	<u>% Sol.</u>	<u>Correct (<math>\mu</math>g/ODml)</u>	<u>Misfold (<math>\mu</math>g/ODml)</u>	<u>Ratio Correct/ Misfold</u>
25	0.10	0.05	0.11	0.01	91	0.0	0.0	-
	0.25	0.12	0.11	0.01	90	0.0	0.0	-
30	0.50	0.25	0.11	0.03	92	3.2	1.8	1.8
	1.00	0.50	0.11	0.05	90	3.1	1.2	2.4
	4.00	2.00	0.11	0.20	94	9.5	2.2	4.3
	8.00	4.00	0.11	0.36	84	9.2	2.1	4.3
	12.00	6.00	0.11	0.36	80	8.6	1.9	4.4
35	0.10	1.00	0.01	0.01	100	0.0	0.0	-
	0.25	1.00	0.01	0.01	100	1.0	0.7	1.3
	0.50	1.00	0.03	0.03	100	4.3	1.7	2.6
	1.00	1.00	0.05	0.05	96	4.2	1.4	3.0
	4.00	1.00	0.22	0.20	91	10.1	2.5	4.0

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	8.00	1.00	0.43	0.03	7	3.9	1.3	3.1
	12.00	1.00	0.65	0.05	7	1.2	0.5	2.4
	1.00	2.00	0.03	0.05	100	4.9	1.4	3.4
	4.00	2.00	0.11	0.21	96	9.5	2.2	4.3
5	8.00	2.00	0.11	0.21	62	8.6	2.0	4.2
	12.00	2.00	0.32	0.05	7	3.5	1.1	3.3

Both the ratio of properly folded to misfolded IGF-I and the yield of properly folded IGF-I increased as the soluble IGF-I concentration was increased to approximately 0.2 mM. Further increases to 0.7 mM (5.3 mg/ml) IGF-I did not significantly decrease either the yield or the properly folded to misfolded ratio. The best yield and ratio were obtained at an IGF-I/DTT ratio of 0.11-0.22.

#### EXAMPLE X

##### Effect of Concentrated and Diluted Urea on Folding

Refractile body IGF-I protein from a 4 OD-ml *E. coli* cell paste, as described in Example 1, was treated with various urea concentrations for 4 hours or 24 hours. The protein to DTT ratio was maintained at 0.11, 20% methanol was added, and the initial protein concentration was increased to be proportional to the initial urea concentration. Four-hour samples were diluted with IGF-I refolding buffer as described in Example IX, except with urea and DTT omitted, to a final urea concentration of 2 M. Then they were incubated for an additional 5 hours before IGF-I levels were measured. IGF-I titers were also measured for the 24-hour samples incubated without urea dilution. The results, shown in Fig. 16, indicate that the refolding yield was lower for samples treated with 4 M or 6 M urea for 4 hours and then diluted and also for samples refolded in 4M or 6M urea for 24 hours (when compared to that obtained with an original 2 M urea concentration). For both diluted and undiluted incubations, the refolding yield decreased as the urea concentration increased.

In summary, the results show that insoluble, misfolded IGF-I isolated from prokaryotic cells can be concurrently solubilized and refolded to the correct conformation in a single buffer containing chaotropic agent and reducing agent. To accomplish this, the chaotropic and reducing agents are kept at the minimal respective concentrations that will substantially solubilize the IGF-I. The yield of correctly folded IGF-I improved with the addition of hydrophobic agent to the buffered solution. Optimal solubilization and refolding are obtained with alkaline buffer at a pH range of 7.5-10.5 with about 2 M urea and about 2-4 mM DTT or 1-2 mM BME.

#### Deposit of Material

The following culture has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

#### Strain

*E. coli* 27C7

#### ATCC Dep. No.

55,244

#### Deposit Date

October 30, 1991

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from the date of deposit. The organism will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if the culture on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANT: GENENTECH, INC.
- (ii) TITLE OF INVENTION: METHOD FOR REFOLDING IGF-I TO ACTIVE CONFORMATION
- 10 (iii) NUMBER OF SEQUENCES: 11
- (iv) CORRESPONDENCE ADDRESS:
- (A) ADDRESSEE: Genentech, Inc.
- 15 (B) STREET: 460 Point San Bruno Blvd
- (C) CITY: South San Francisco
- (D) STATE: California
- (E) COUNTRY: USA
- (F) ZIP: 94080-4990
- 20 (v) COMPUTER READABLE FORM:
- (A) MEDIUM TYPE: 5.25 inch, 360 Kb floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- 25 (D) SOFTWARE: patin (Genentech)
- (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE: 03 December 1992
- 30 (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- 35 (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Hasak, Janet E.
- (B) REGISTRATION NUMBER: 28,616
- (C) REFERENCE/DOCKET NUMBER: 729
- 40 (ix) TELECOMMUNICATION INFORMATION:
- (A) TELEPHONE: 415/225-1896
- (B) TELEFAX: 415/952-9881
- (C) TELEX: 910/371-7168

## 45 (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 55 bases
- 50 (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

55 GGTCCCGAAA CTCTGTGCGG TGCTGAACTG GTTGACGCTC TGCAGTTTGT 50  
TTGCG 55

## (2) INFORMATION FOR SEQ ID NO:2:

- 60 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 64 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear
- 65 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTCACCGCAA ACAAACTGCA GAGCGTCAAC CAGTTCAGCA CCGCACAGAG 50

TTTCGGGACC TGCA 64

## (2) INFORMATION FOR SEQ ID NO:3:

- 5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 84 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTAGAATTAT GATGATTACT CTGCGCAAAC TTCCTCTGGC GGTGCGCGTC 50  
GCAGCGGGCG TAATGTCTGC TCAGGCCATG GCCA 84

## 15 (2) INFORMATION FOR SEQ ID NO:4:

- 20 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 84 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## 25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GATCTGGCCA TGGCCTGAGC AGACATTACG CCCGCTGCGA CGGCAACCGC 50  
CAGAGGAAGT TTGCGCAGAG TAATCATCAT AATT 84

## 30 (2) INFORMATION FOR SEQ ID NO:5:

- 35 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 59 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

40 GGCCGGTCCC GAAACTCTGT GCGGTGCTGA ACTGGTTGAC GCTCTGCAGT 50  
TTGTTTGCG 59

## (2) INFORMATION FOR SEQ ID NO:6:

- 45 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 60 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## 50 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTCACCGCAA ACAAACTGCA GAGCGTCAAC CAGTTCAGCA CCGCACAGAG 50  
TTTCGGGACC 60

## 55 (2) INFORMATION FOR SEQ ID NO:7:

- 60 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 50 bases  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

65 GGCCACTCTG TGCGGTGCTG AACTGGTTGA CGCTCTGCAG TTTGTTTGCG 50

## (2) INFORMATION FOR SEQ ID NO:8:

-35-

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 51 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTCACGCAA ACAAACTGCA GAGCGTCAAC CAGTTCAGCA CCGCACAGAG 50

T 51

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 39 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCTAACGCTC GGTGCGGCC GGGCGTTTTT TATTGTAA 39

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 39 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTAACAATAA AAAACGCCCG GCGGCAACCG AGCGTTAGG 39

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 757 bases  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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 TCATTGCTGA GTTGTTATTT AAGCTTGCCC AAAAAGAAGA AGAGTCGAAT 100  
 GAAGTGTGTG CGCAGGTAGA AGCTTTGGAG ATTATCGTCA CTGCAATGCT 150  
 TCGCAATATG GCGCAAAATG ACCAACAGCG GTTGATTGAT CAGGTAGAGG 200  
 GGGCGCTGTA CGAGGTAAAG CCCGATGCCA GCATTCCCTGA CGACGATACG 250  
 GAGCTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCAGTA 300  
 AAAAGTTAAT CTTTCAACA GCTGTCATAA AGTTGTCACG GCCGAGACTT 350  
 ATAGTCGCTT TGTTTTATT TTTAATGTA TTTGTAAC TA GTACGCAAGT 400  
 TCACGTAAAA AGGGTATCTA GAATT ATG ATG ATT ACT CTG CGC 443  
 Met Met Ile Thr Leu Arg  
 1 5

AAA CTT CCT CTG GCG GTT GCC GTC GCA GCG GGC GTA ATG 482  
 Lys Leu Pro Leu Ala Val Ala Val Ala Ala Gly Val Met  
 10 15

TCT GCT CAG GCC ATG GCC GGT CCC GAA ACT CTG TGC GGT 521  
 Ser Ala Gln Ala Met Ala Gly Pro Glu Thr Leu Cys Gly  
 20 25 30

GCT GAA CTG GTT GAC GCT CTG CAG TTT GTT TGC GGT GAC 560  
 Ala Glu Leu Val Asp Ala Leu Gln Phe Val Cys Gly Asp

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				35				40				45	
	CGT	GGT	TTT	TAT	TTT	AAC	AAA	CCC	ACT	GGT	TAT	GGT	TCT
	Arg	Gly	Phe	Tyr	Phe	Asn	Lys	Pro	Thr	Gly	Tyr	Gly	Ser
5					50					55			599
	TCT	TCT	CGT	CGT	GCT	CCC	CAG	ACT	GGT	ATT	GTT	GAC	GAA
	Ser	Ser	Arg	Arg	Ala	Pro	Gln	Thr	Gly	Ile	Val	Asp	Glu
		60					65					70	638
10	TGC	TGC	TTT	CGT	TCT	TGC	GAC	CTG	CGT	CGT	CTG	GAA	ATG
	Cys	Cys	Phe	Arg	Ser	Cys	Asp	Leu	Arg	Arg	Leu	Glu	Met
				75				80					677
15	TAT	TGC	GCT	CCC	CTG	AAA	CCC	GCT	AAA	TCT	GCT		710
	Tyr	Cys	Ala	Pro	Leu	Lys	Pro	Ala	Lys	Ser	Ala		
	85					90					95		

TAGAAGCTCC TAACGCTCGG TTGCCGCCGG GCGTTTTTAA TTGTAA 757

## WHAT IS CLAIMED IS:

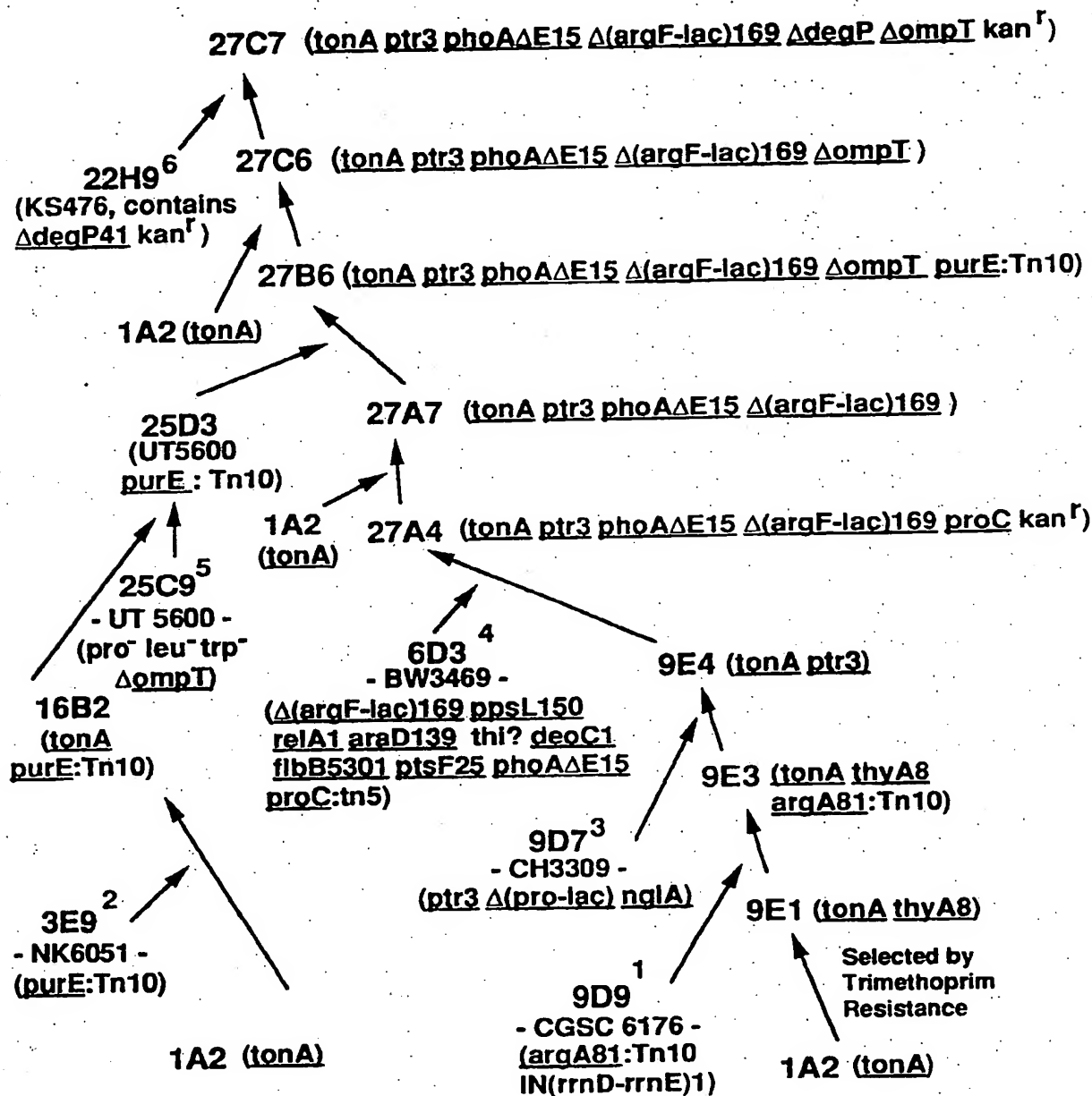
1. A process for reactivating insoluble, misfolded IGF-I contained in prokaryotic host cells, which process comprises:
  - (a) isolating said IGF-I; and
  - (b) incubating said isolated IGF-I in an alkaline buffered solution comprising no more than 3 M chaotropic agent and the minimal concentration of reducing agent sufficient for solubilization, under conditions of IGF-I concentration and incubation temperature and time whereby solubilization, unfolding, and refolding of the IGF-I all occur during the incubation.
2. The process of claim 1 wherein the IGF-I is isolated from the periplasm of the host cells.
3. The process of claim 1 wherein the concentration of IGF-I is about 0.5-5.5 mg IGF-I per ml.
4. The process of claim 1 wherein said buffered solution has a pH of at least about 7.5.
5. The process of claim 1 wherein said buffered solution has a pH of about 7.5-10.5.
6. The process of claim 5 wherein said buffered solution comprises Capso buffer at about pH 8.5-10.5.
7. The process of claim 1 wherein the incubation step is carried out at about 10-40°C for at least about 1 hour.
8. The process of claim 1 wherein the incubation step is carried out at about 15-37°C for at least about 1 hour.
9. The process of claim 1 wherein said chaotropic agent is urea at 1-3 M or guanidine hydrochloride at about 1 M.
10. The process of claim 1 wherein said reducing agent is dithiothreitol at about 1-8 mM,  $\beta$ -mercaptoethanol at about 0.2-2 mM, or cysteine.
11. The process of claim 1 wherein said chaotropic agent is urea at about 1.5-2.5 M and said reducing agent is dithiothreitol at about 2-4 mM or  $\beta$ -mercaptoethanol at about 1-2 mM.
12. The process of claim 1 wherein said buffered solution additionally comprises a hydrophobic agent.
13. The process of claim 12 wherein said hydrophobic agent is methanol or ethanol.
14. The process of claim 1 wherein step (a) is carried out by treating the host cell culture with a buffered solution of ionic strength sufficient to substantially solubilize the host polypeptides, but not the insoluble, misfolded IGF-I, disrupting the cells to form a soluble fraction and a fraction containing the insoluble, misfolded IGF-I, centrifuging the disrupted cells, and collecting the pellet containing the insoluble, misfolded IGF-I fraction.



15. The process of claim 14 wherein the cells are treated by suspension in the buffered solution at about pH 5 to 9 and at an ionic strength of about 0.01 to 2 M.
16. The process of claim 15 wherein the buffered solution in which the cells are suspended is at about pH 6 to 8 and has an ionic strength of 0.1 to 0.2 M.
17. The process of claim 1 wherein said host cells are bacterial cells.
18. The process of claim 17 wherein said bacterial cells are *E. coli*.
19. The process of claim 18 wherein the *E. coli* cells are deficient in an endogenous protease.
20. The process of claim 19 wherein the *E. coli* cells have the complete genotype of *E. coli* 27C7.

## FIG. 1

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Lineage of *E. coli* W3110 Strain 27C7

1. Obtained from B. Bachman, E.coli Genetic Stock Center
2. From Dr. Nancy Kleckner's Laboratory, similar strain available from Dr. Carol Gros, University of Wisconsin
3. J.Bact. 140, 1979, p. 125.
4. Obtained from Professor Barry Wanner, Purdue University.
5. J.Bact. 153, 1983, p. 1104-1106.
6. Obtained from Harvard Medical School, Office of Technology Licensing and Industry-Sponsored Research.

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FIG. 2A

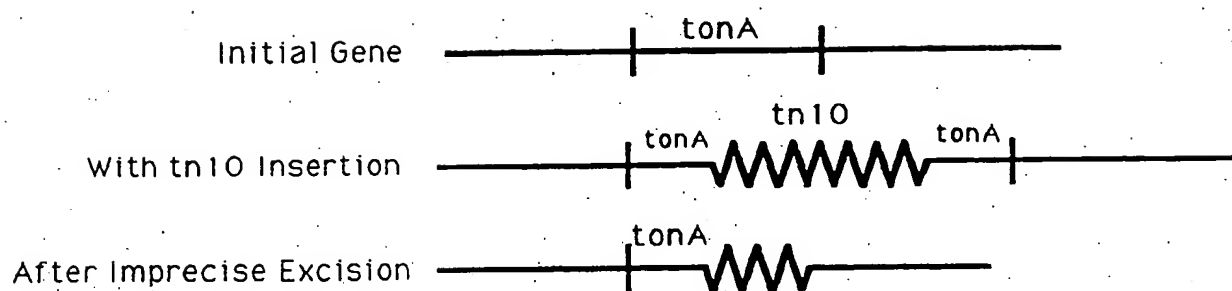


FIG. 2B

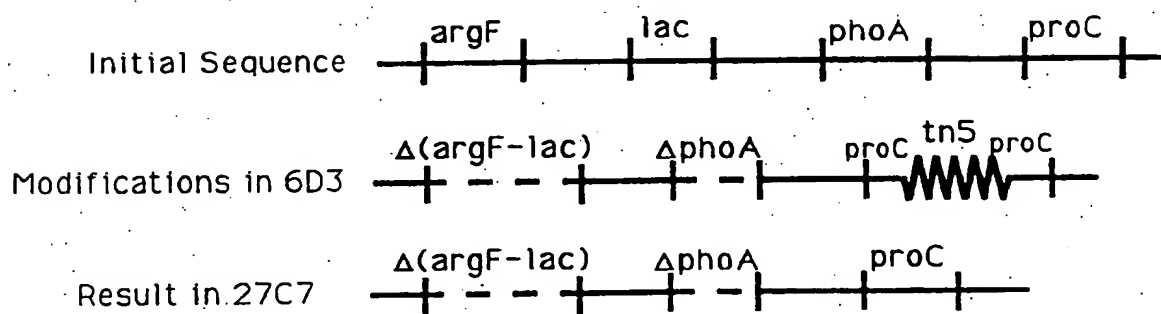
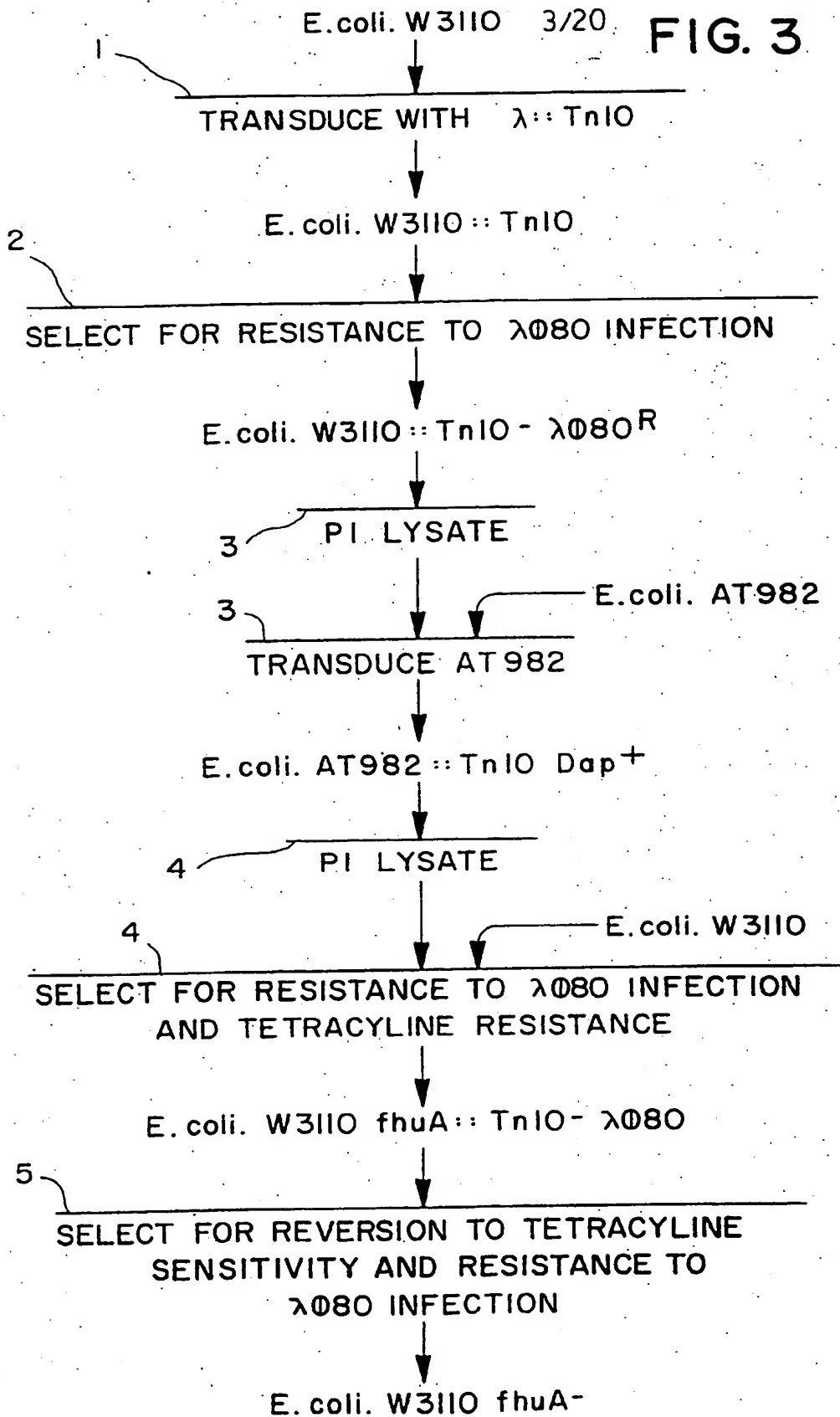


FIG. 3



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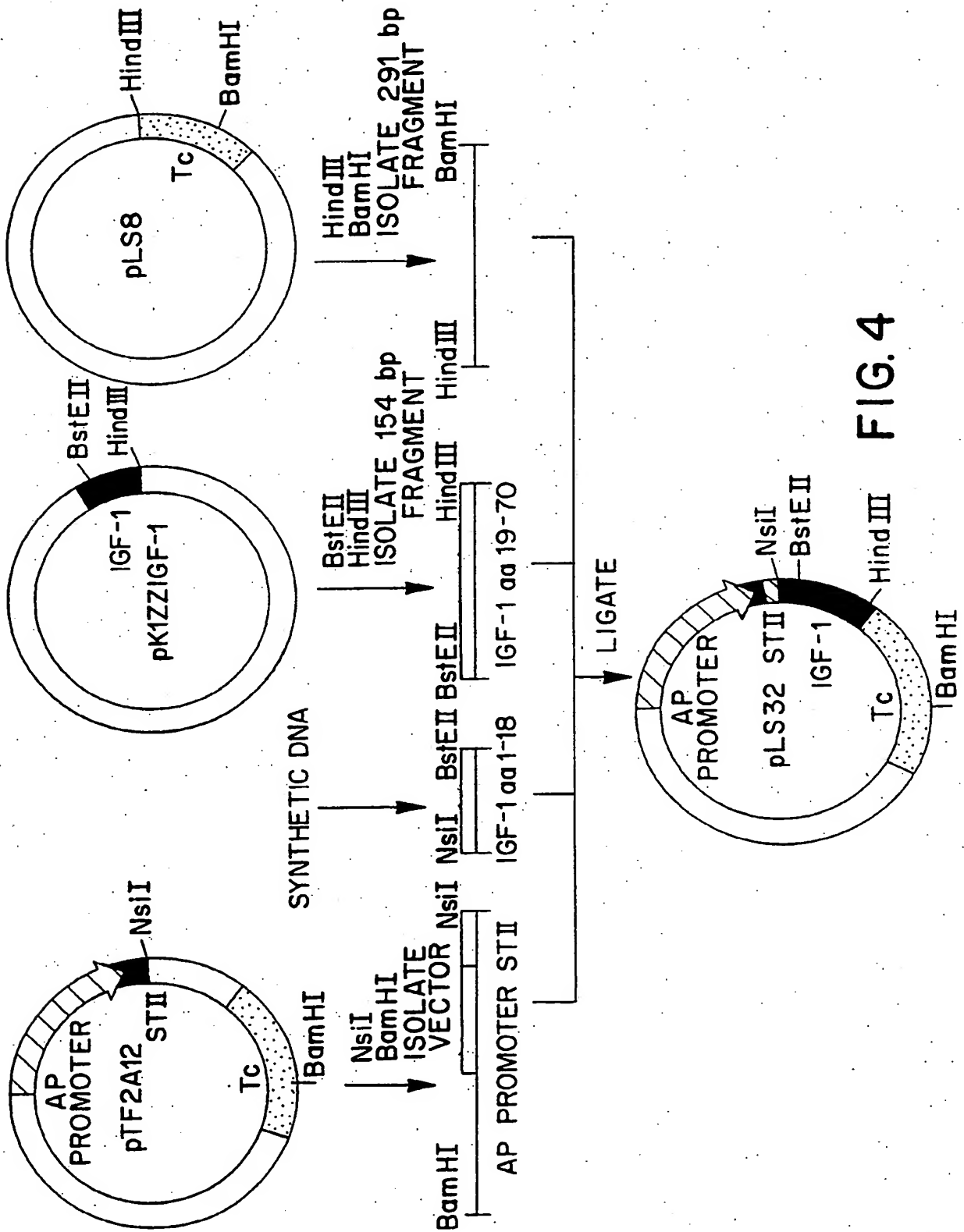
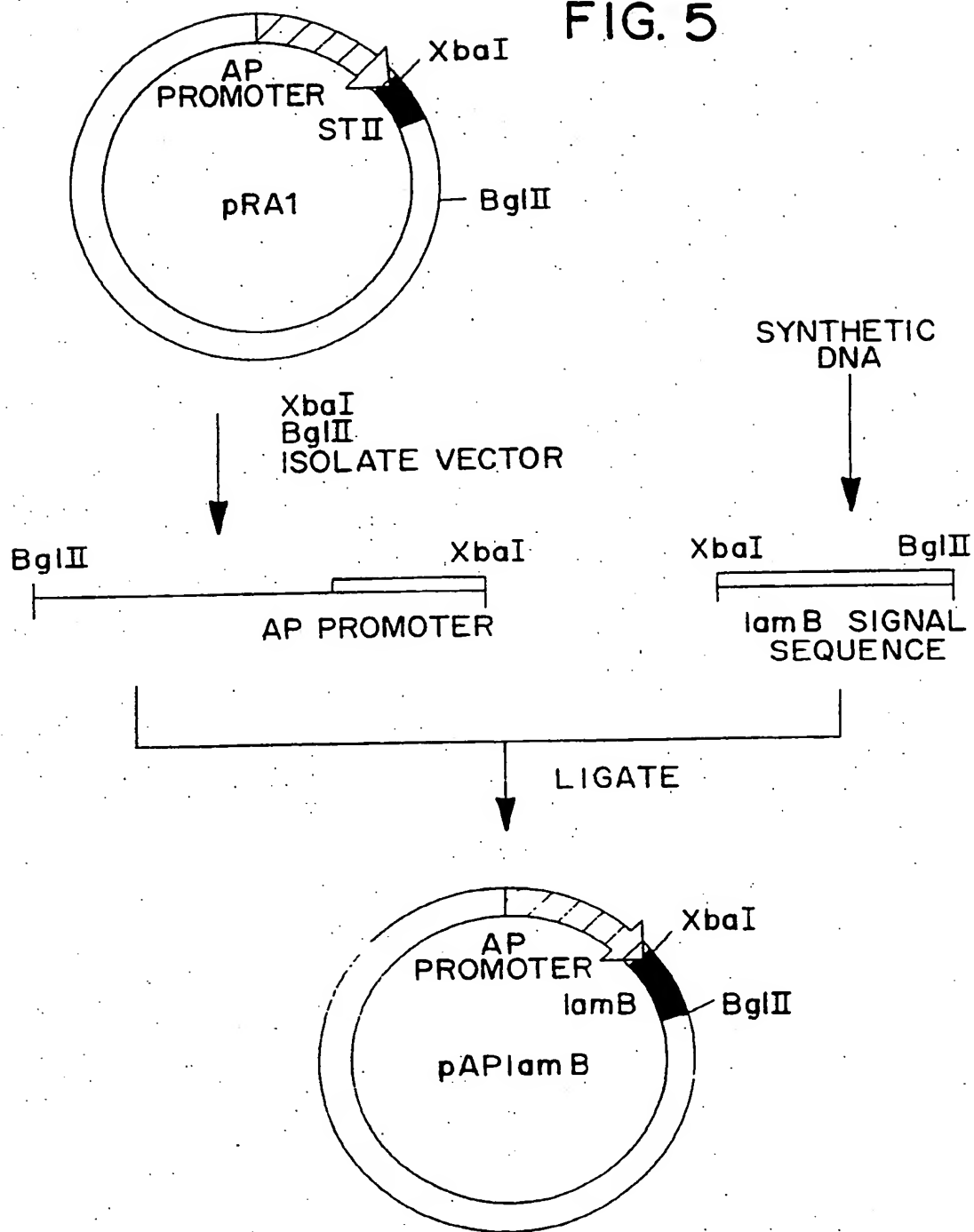


FIG. 4

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FIG. 5



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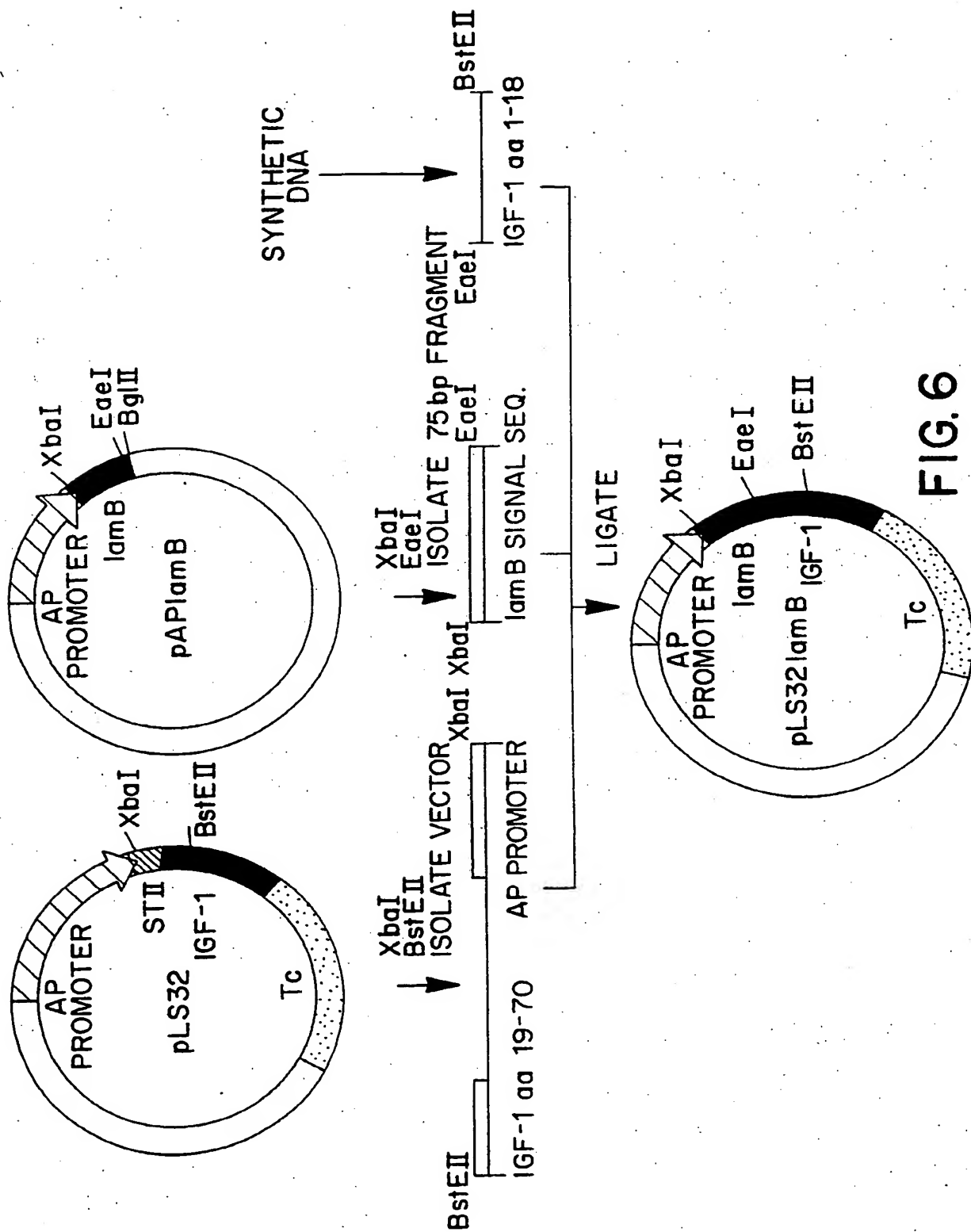
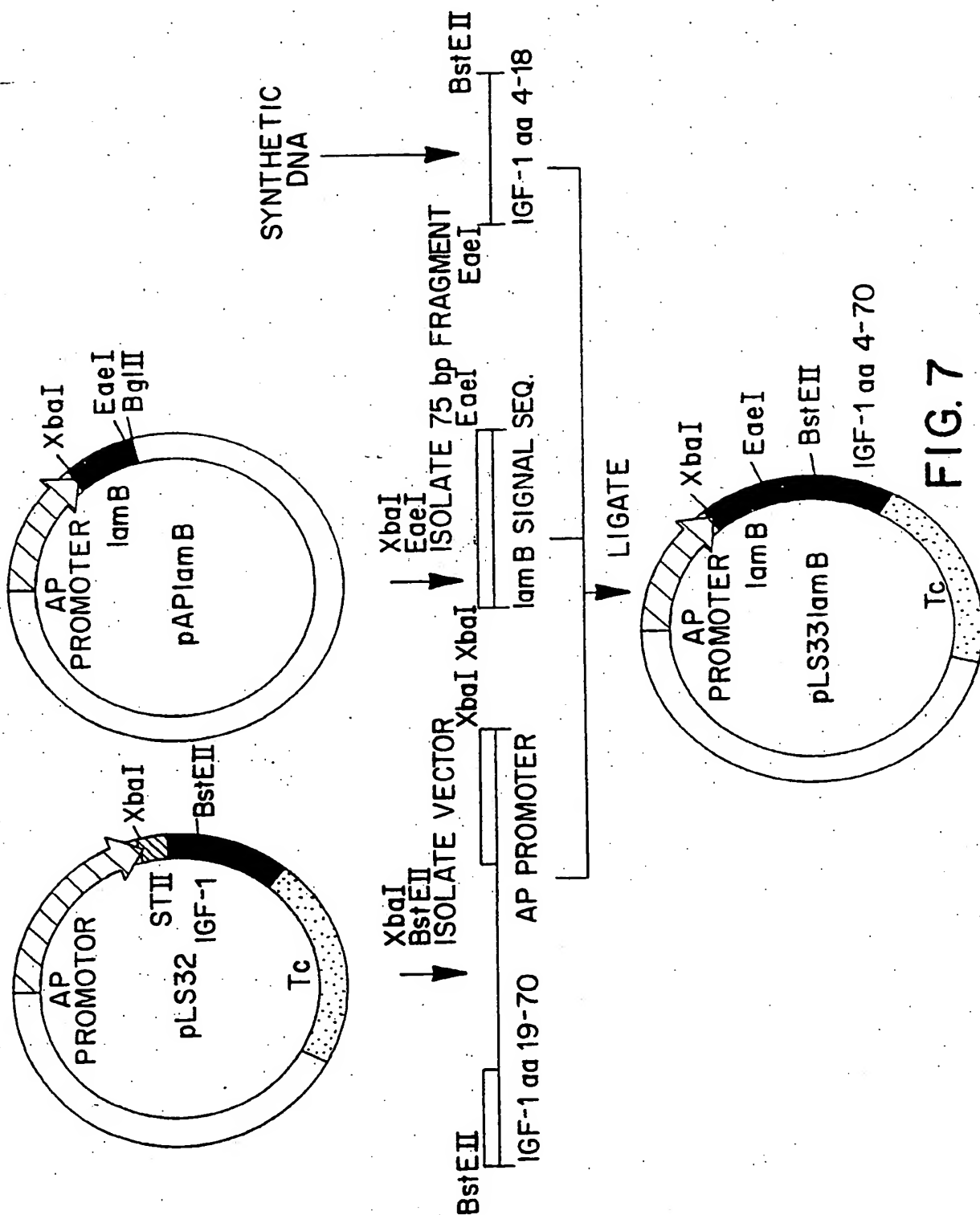


FIG. 6

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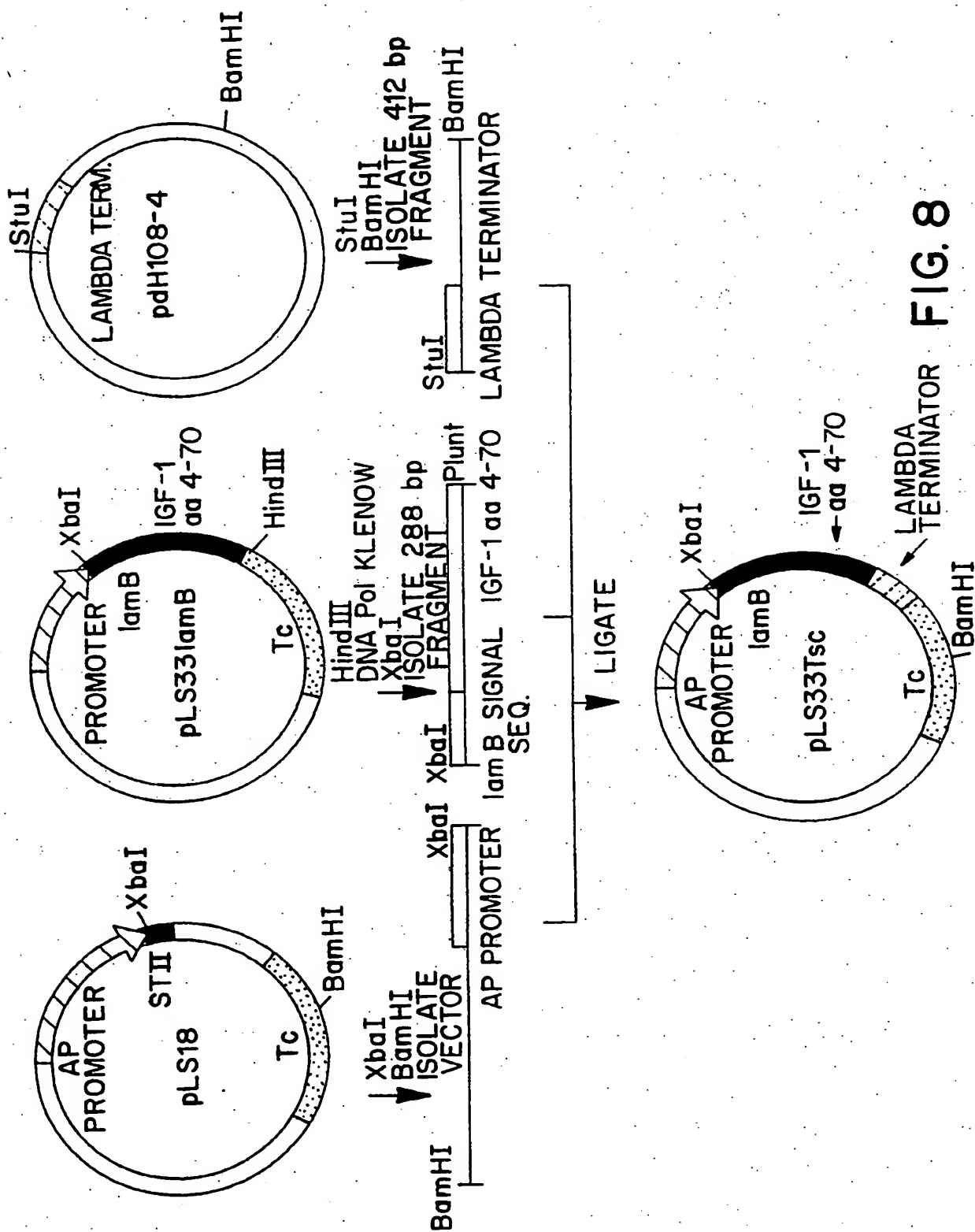


FIG. 8

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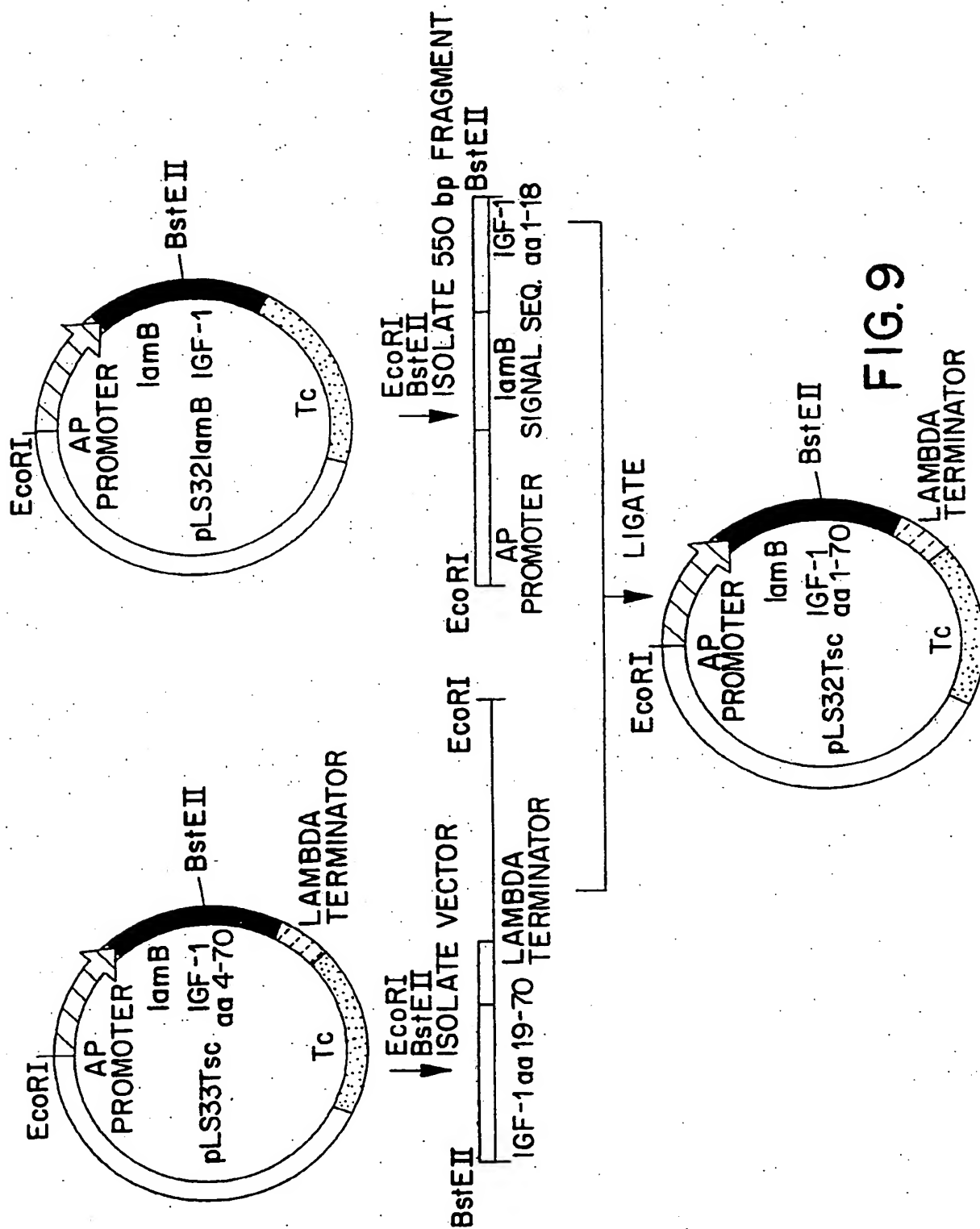


FIG. 9

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GAATTCAACT TCTCCATACT TTGGATAAGG AAATACAGAC ATGAAAAATC 50  
TCATTGCTGA GTTGTTATTT AAGCTTGCCC AAAAAGAAGA AGAGTCGAAT 100  
GAACTGTGTG CGCAGGTAGA AGCTTTGGAG ATTATCGTCA CTGCAATGCT 150  
TCGCAATATG GCGCAAAATG ACCAACACGG GTTGATTGAT CAGGTAGAGG 200  
GGGCGCTGTA CGAGGTAAAG CCCGATGCCA GCATTCCTGA CGACGATACG 250  
GAGCTGCTGC GCGATTACGT AAAGAAGTTA TTGAAGCATC CTCGTCAGTA 300  
AAAAGTTAAT CTTTTCACAA GCTGTCATAA AGTTGTCACG GCCGAGACTT 350  
ATAGTCGCTT TGTTTTTATT TTTTAATGTA TTTGTAAGTA GTACGCAAGT 400

FIG. 10A

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TCACGTAAAA AGGGTATCTA GAATTATG ATG ATT ACT CTG CGC 443  
 Met Ile Thr Leu Arg  
 -24

AAA CTT CCT CTG GCG GTT GCC GTC GCA GCG GGC GTA ATG 482  
 Lys Leu Pro Leu Ala Val Ala Val Ala Gly Val Met  
 -10

TCT GCT CAG GCC ATG GCC GGT CCC GAA ACT CTG TGC GGT 521  
 Ser Ala Gln Ala Met Ala Gly Pro Glu Thr Leu Cys Gly  
 1

GCT GAA CTG GTT GAC GCT CTG CAG TTT GTT TGC GGT GAC 560  
 Ala Glu Leu Val Asp Ala Leu Gln Phe Val Cys Gly Asp  
 10 20

CGT GGT TTT TAT TTT AAC AAA CCC ACT GGT TAT GGT TCT 599  
 Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly Ser  
 30

FIG. 10B

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TCT TCT CGT CGT GCT CCC CAG ACT GGT ATT GTT GAC GAA 638  
Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp Glu

40

TGC TGC TTT CGT TCT TGC GAC CTG CGT CGT CTG GAA ATG 677  
Cys Cys Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Met

50

TAT TGC GCT CCC CTG AAA CCC GCT AAA TCT GCT TAG AAGCTC 719  
Tyr Cys Ala Pro Leu Lys Pro Ala Lys Ser Ala AM\*

70

60

CTAACGCTCGG TTGCCGCCCGG GCGTTTTTTA TTGTTAA 757

Position Number -24 signifies the start of the lamB signal sequence.  
Position Number 1 signifies the start of the IGF-I protein.

**FIG. 10C**

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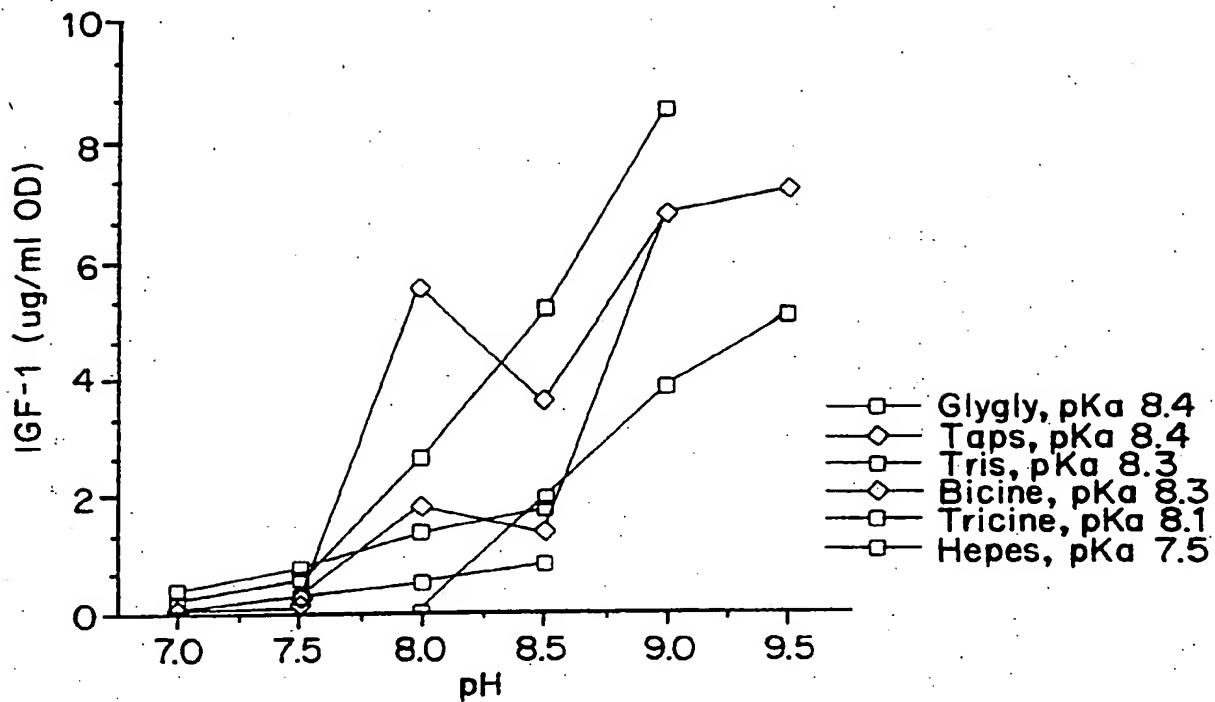


FIG. IIA

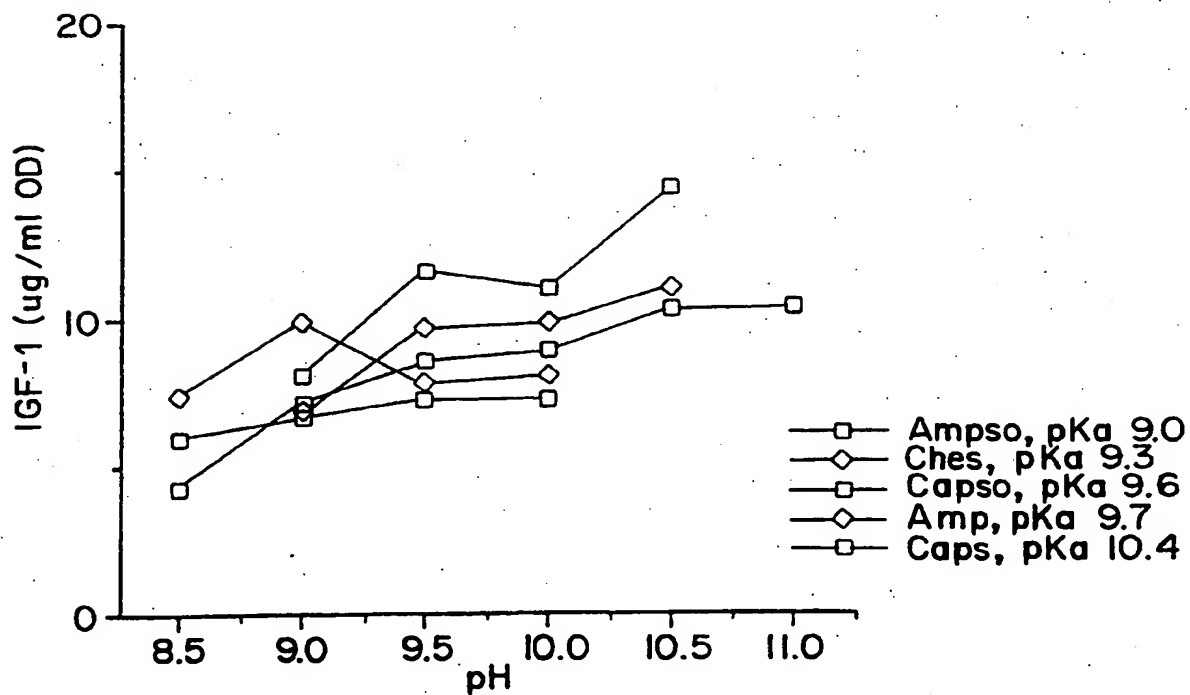


FIG. IIB

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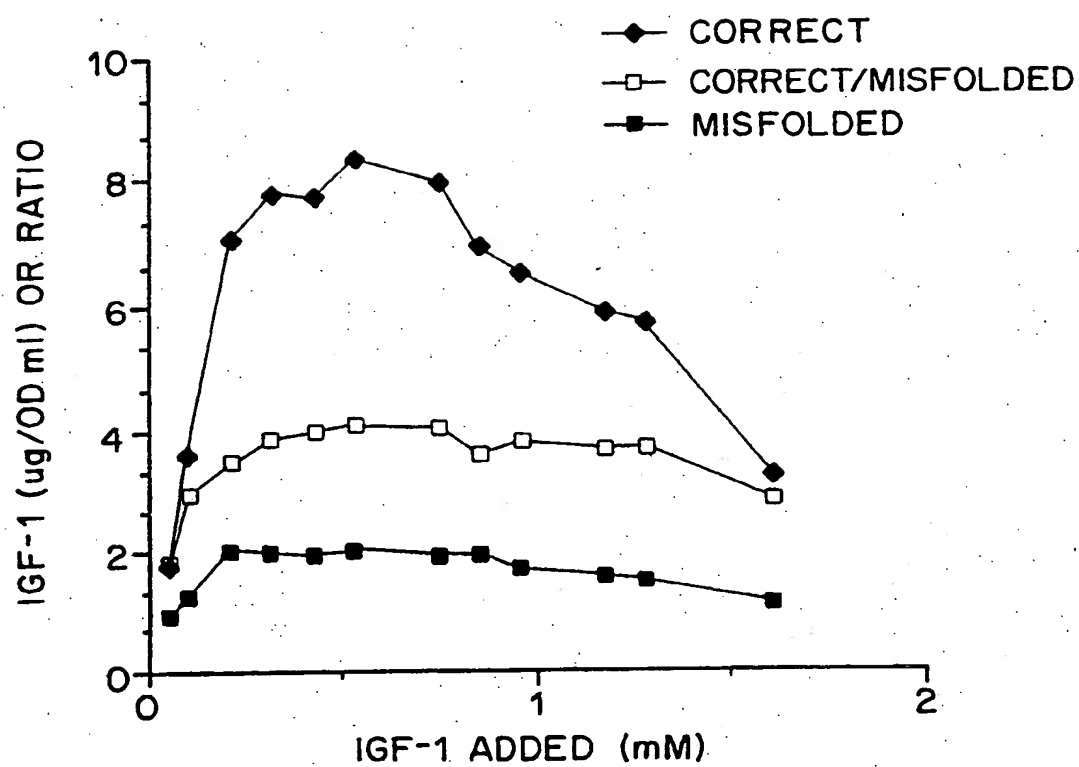


FIG. 12

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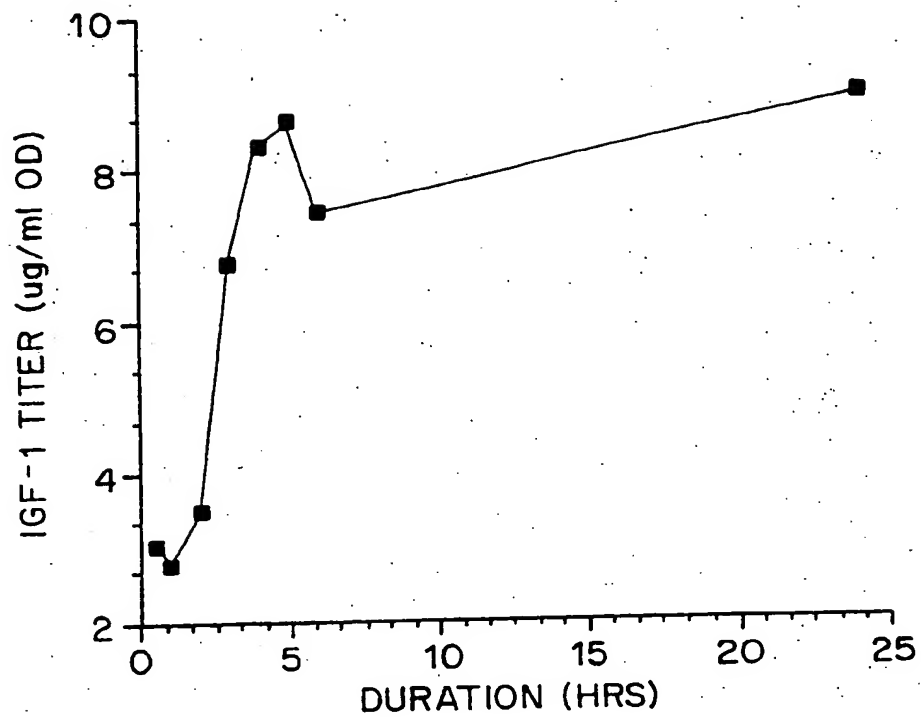


FIG. 13A

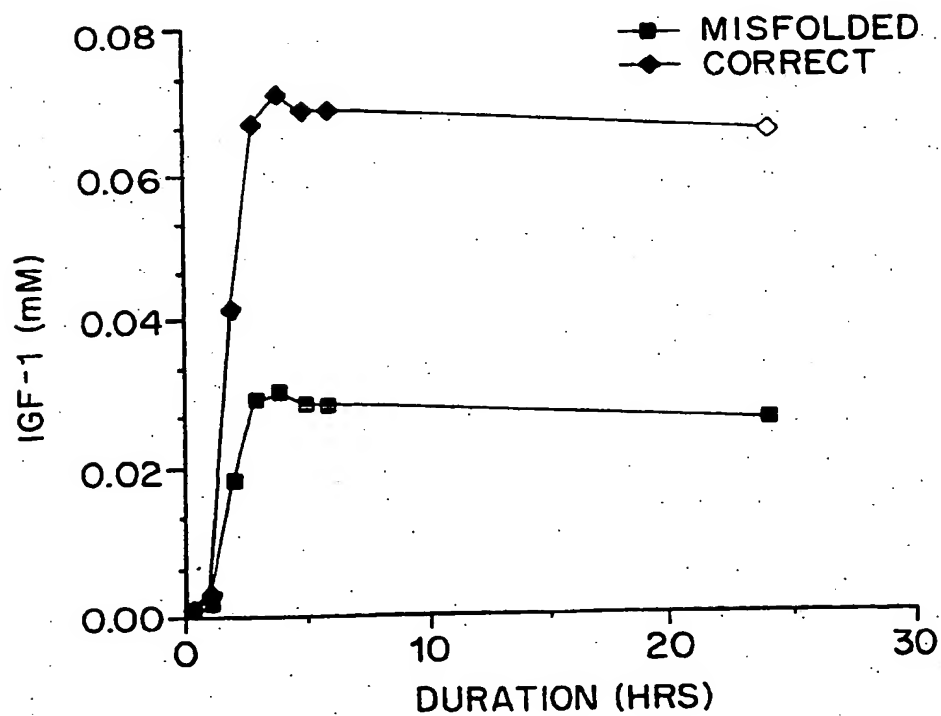


FIG. 13B



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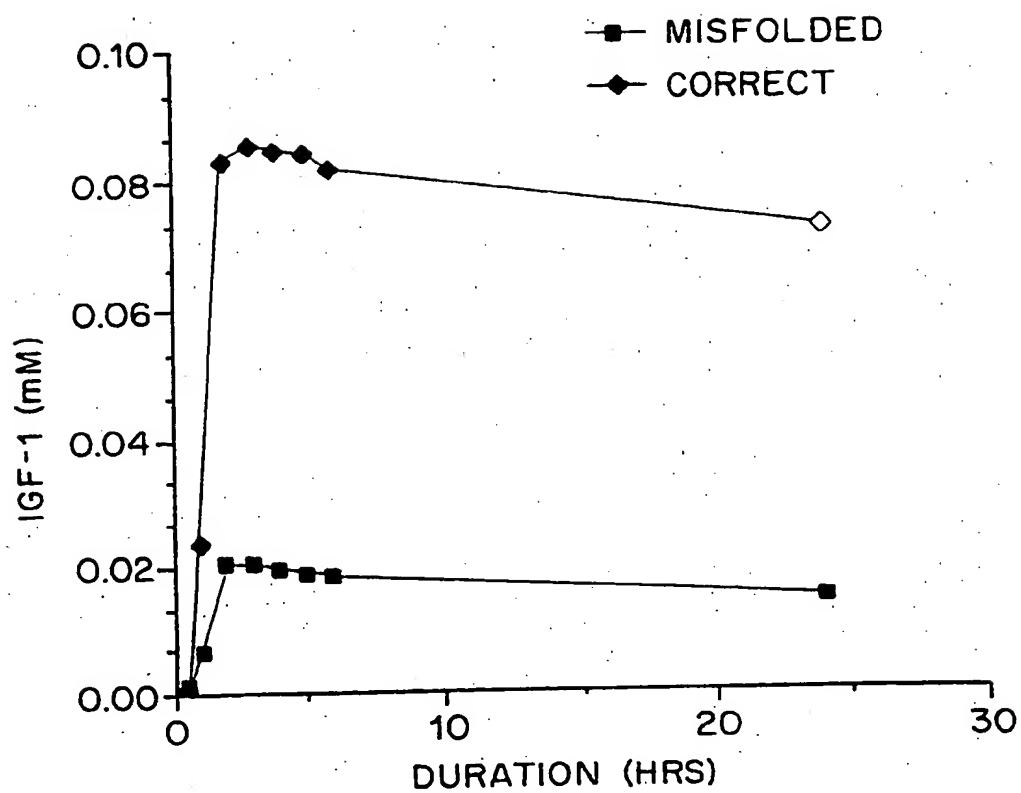
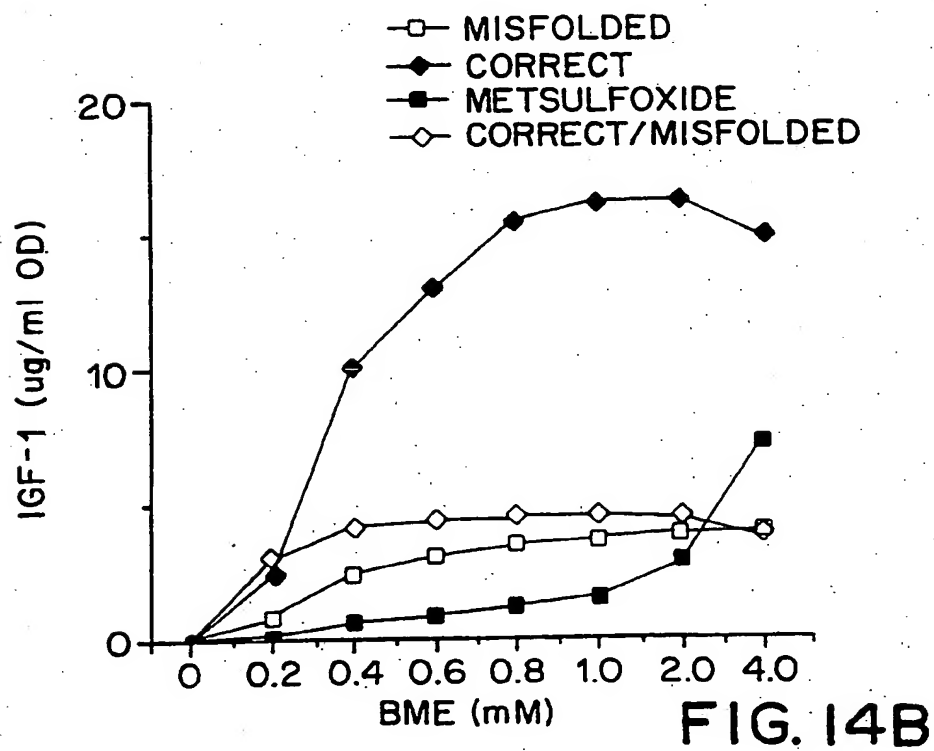
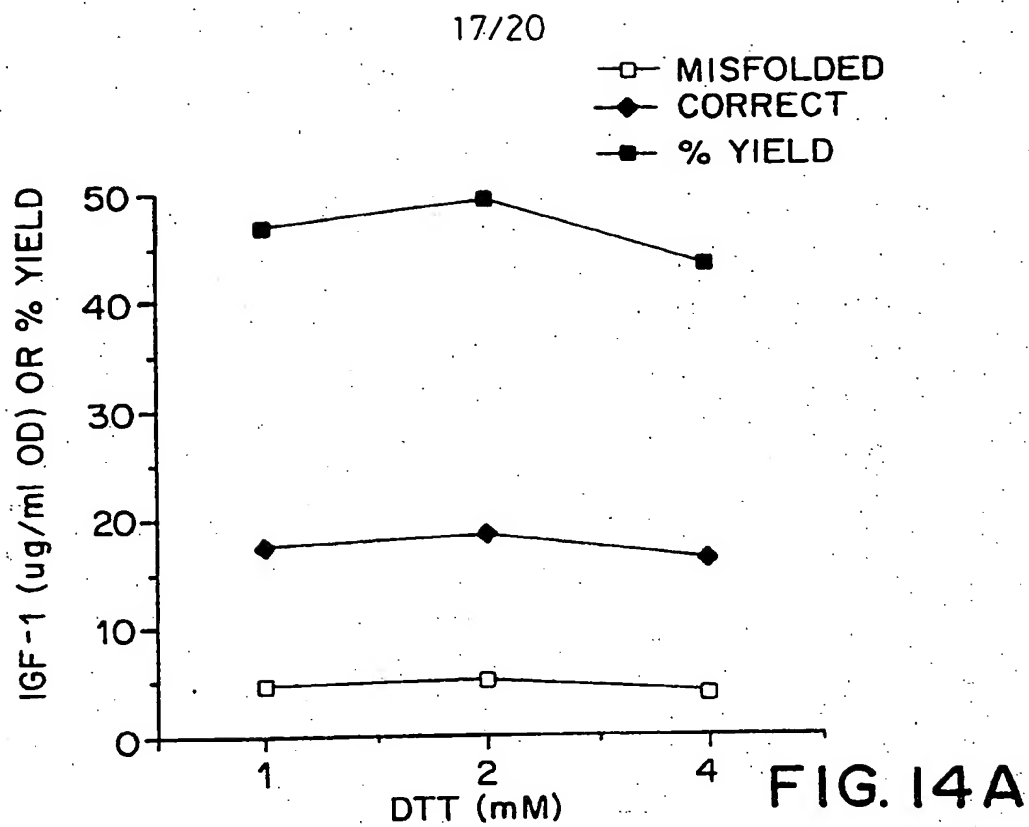


FIG. 13C



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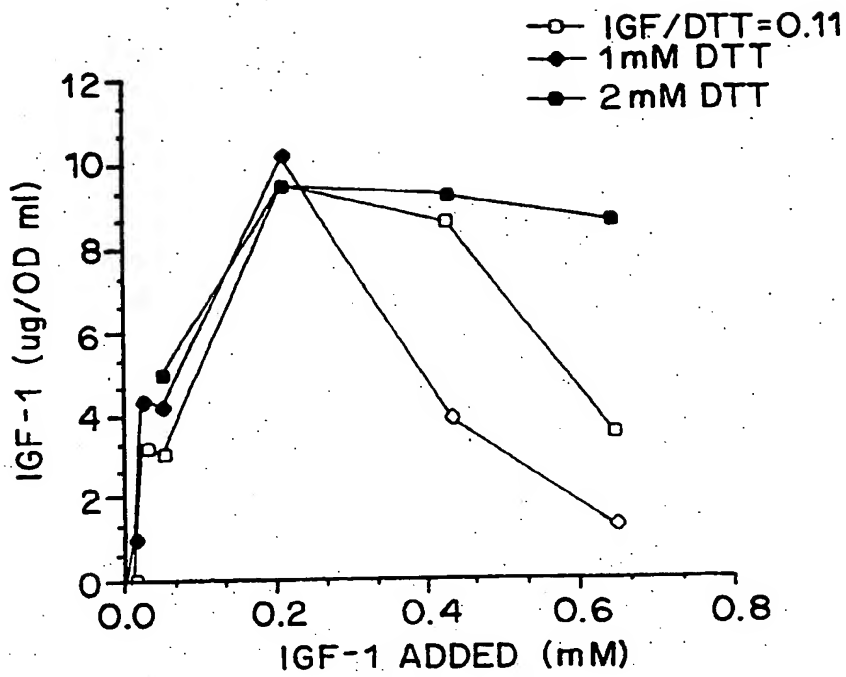


FIG. 15A

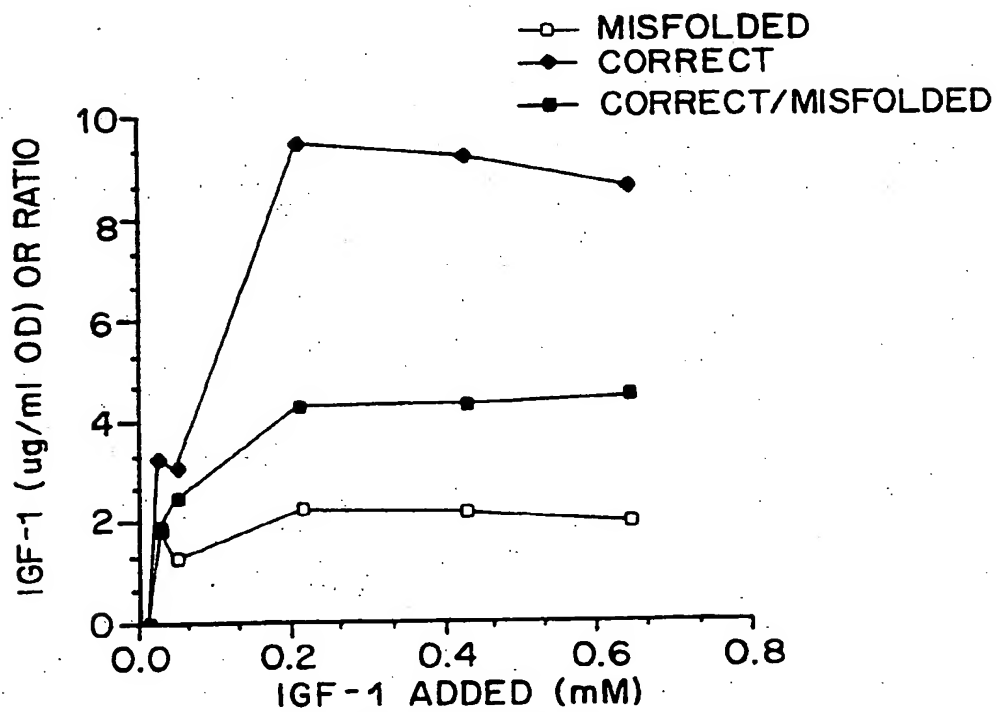


FIG. 15B

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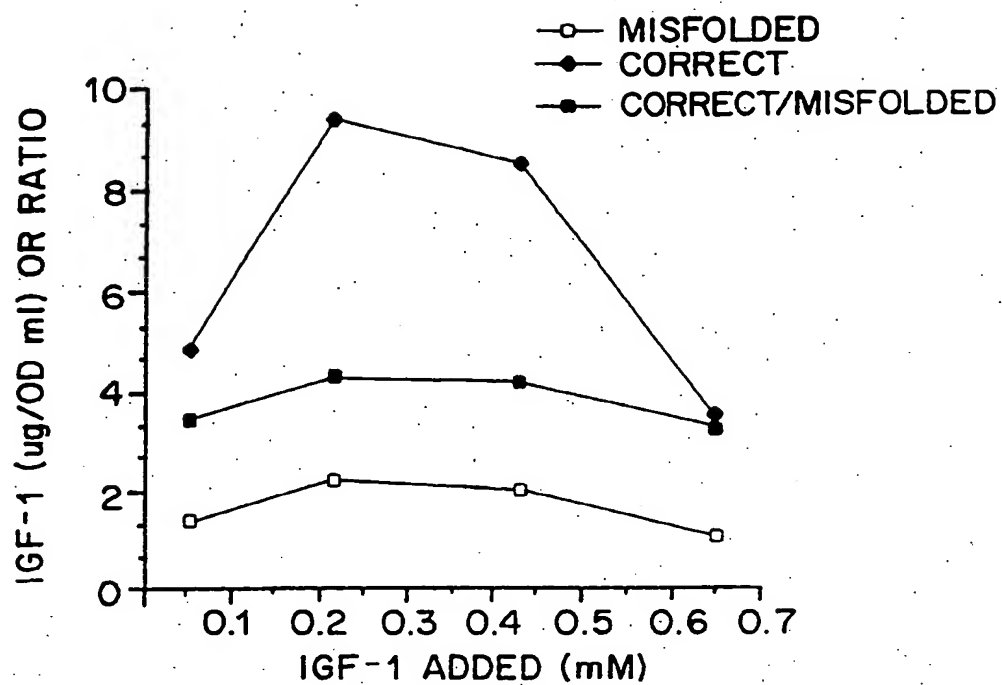


FIG. 15C

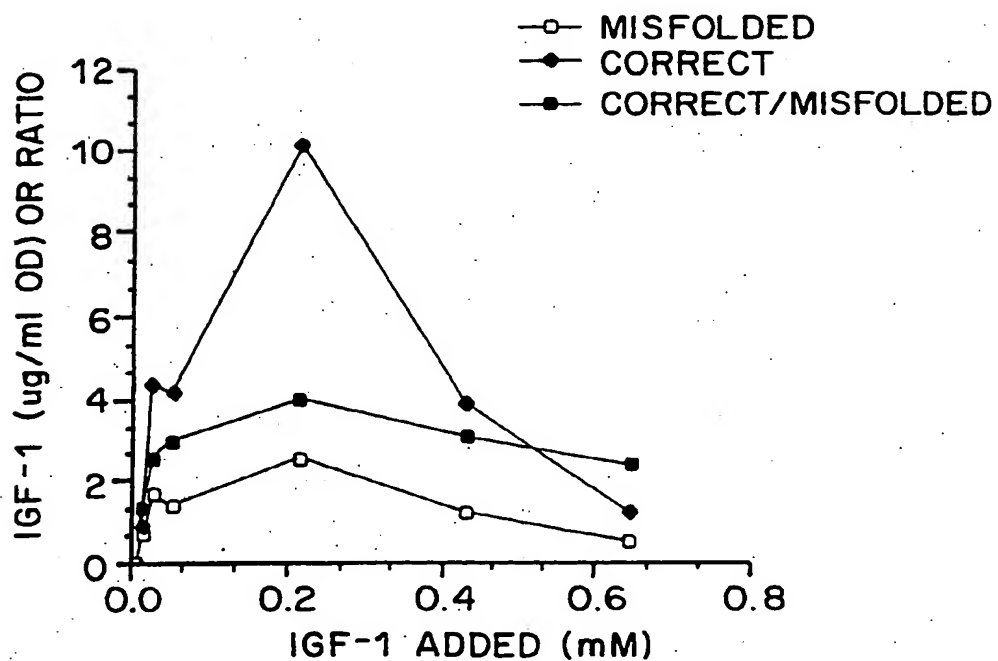


FIG. 15D

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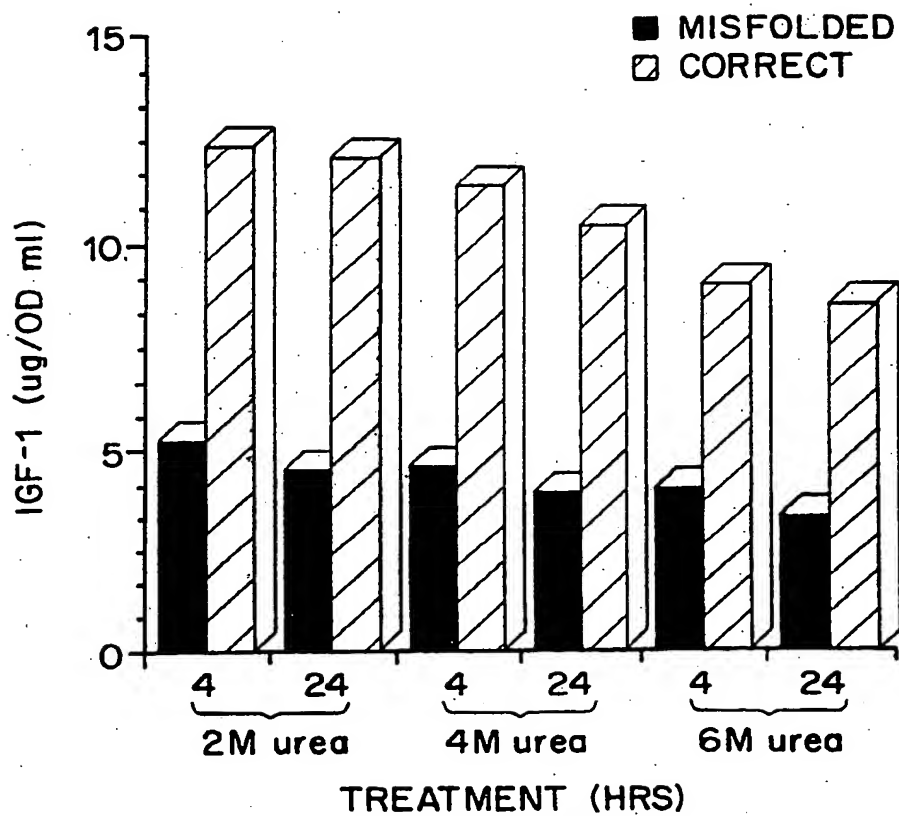


FIG. 16

## INTERNATIONAL SEARCH REPORT

PCT/US 92/10625

International Application No

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/16; C07K3/08; C12N1/21; //(C12N1/21, C12R1:19)		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C07K ; C12N ; C12P	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
A	EP,A,0 264 074 (FUGISAWA PHARMACEUTICAL CO.,LTD.) 20 April 1988 cited in the application see page 37, line 13 - line 26	1,17-18
A	NUCLEIC ACIDS RESEARCH. vol. 13, no. 6, 1985, ARLINGTON, VIRGINIA US pages 1923 - 1938 GARY BUELL ET AL 'Optimizing the expression in E. coli of a synthetic gene encoding Somatomedin-C (IGF-I)' cited in the application see the whole document	1,17-20
-/--		
<p><sup>10</sup> Special categories of cited documents : <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
15 APRIL 1993	10. 05. 93	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	LE CORNEC N.D.R.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		Relevant to Claim No.
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	
A	ANNALS NEW YORK ACADEMY OF SCIENCES 1986, pages 31 - 52 MINEO NIWA ET AL 'chemical synthesis, Cloning and Expression of genes for human Somatomedin C (Insulin-like-Growth -Factor_I) and 59Val-somatomedin C' see page 44	1,17-18
P,X	ABSTRACTS PAPER OF THE AMERICAN CHEMICAL SOCIETY vol. 203, no. 1-3, April 1992, JUDY Y. CHANG ET AL 'Folding of IGF-I' * see the abstract BIOT 117 *	1-20

US 9210625  
SA 68295

15/04/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0264074	20-04-88	DE-A- 3783305 JP-A- 63233791	11-02-93 29-09-88
-----			

## УСЛОВИЯ ПОСЫЛКИ

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82